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Original article

Novel imidazole derivatives as heme oxygenase-1 (HO-1) and heme oxygenase-2 (HO-2) inhibitors and their cytotoxic activity in human-derived cancer cell lines

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ABSTRACT

Heme oxygenase (HO) is a cytoprotective enzyme that can be overexpressed in some pathological conditions, including certain cancers. In this work, novel imidazole derivatives were designed and synthesized as inhibitors of heme oxygenase-1 (HO-1) and heme oxygenase-2 (HO-2). In these compounds the imidazole ring, crucial for the activity, is connected to a hydrophobic group, represented by aryloxy, benzothiazole, or benzoxazole moieties, by means of alkyl or thioalkyl chains of different length. Many of the tested compounds were potent and/or selective against one of the two isoforms of HO. Furthermore, most of the pentyl derivatives showed to be better inhibitors of HO-2 with respect to HO-1, revealing a critical role of the alkyl chain in discriminating between the two isoenzymes. Compounds which showed the better profile of HO inhibition were selected and tested to evaluate their cytotoxic properties in prostate and breast cancer cell lines (DU-145, PC3, LnCap, MDA-MB-231, and MCF-7). In these assays, aryloxyalkyl derivatives resulted more cytotoxic than benzothiazolethioalkyl ones; in particular compound **31** was active against all the cell lines tested, confirming the anti-proliferative properties of HO inhibitors and their potential use in the treatment of specific cancers.

1. Introduction

Heme oxygenase (HO) is the rate-limiting enzyme that catalyzes the metabolism of the pro-oxidant agent heme into equimolar amounts of carbon monoxide (CO), free iron, and the bile pigment biliverdin, which in turn is reduced to bilirubin by biliverdin reductase [1]. Each of these metabolites has regulatory functions: CO is a cell-signaling molecule with anti-inflammatory, anti-proliferative, and antiapoptotic effects [2], biliverdin and bilirubin possess antioxidant properties [3].

Three isoforms of HO have been identified so far, namely HO-1, HO-2, and HO-3 [4], but enzymatic activity is attributable to two functional isoforms only: HO-1, which is highly inducible and HO-2, which is constitutive. HO-1 is a 32 kDa heat shock protein that is expressed in tissues rich in reticuloendothelial cells such as spleen. In other tissues its expression is induced by numerous stimuli, including its substrate heme, heavy metals, reactive oxygen species, hypoxia, NO, ultraviolet radiations, and xenobiotics. HO-2 is a 36 kDa protein constitutively present in brain and testis, but also in endothelium, distal nephron segment, liver, and gut myenteric plexus.

The biological functions of HO are mainly associated with a basic adaptive and defensive response against oxidative and cellular stress and with a maintaining of cellular homeostasis [5]. Consequently, HO-1 up-regulation might have therapeutic applications in many oxidative stress-associated diseases, such as diabetes, obesity, cardiovascular and eye diseases, atherosclerosis, inflammation, and vascular injury. However, over-activity of the HO

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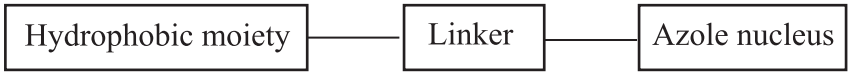
system may not exert only cytoprotective effects, but may contribute to tissue injury under certain unfavorable cases. HO-1 down-regulation or direct inhibition may ameliorate diseases in which HO over-activity is observed, for example hyperbilirubinemia and neonatal jaundice [6], and various bacterial or fungal infectious diseases [7]. Moreover, an increased expression of HO-1 has been observed in several cancers, suggesting that one of the main therapeutic applications of HO-1 inhibitors could be the treatment of specific cancers. Multiple studies confirm implications of HO in various tumors [8,9] and the effectiveness of treatment with HO inhibitors [10,11].

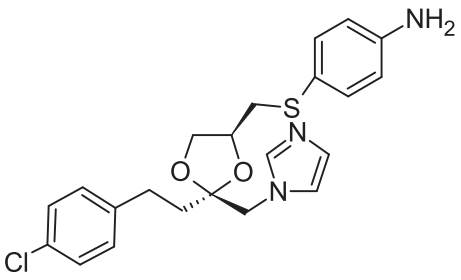
Most of the actual knowledge on HO system is related to HO-1 isoform, whereas spare information is available on HO-2 isoform. Recent works report significant tumor regression mediated by a down regulation of both HO-1 and HO-2 system [12]. Therefore, selective HO-1 or HO-2 inhibitors may provide useful tools for the elucidation of the physiological roles of HO system and may have important clinical applications.

In the recent years, a number of imidazole-based compounds derived from structural modifications of the first described non-porphyrin HO-inhibitor azalanstat **1** (Fig. 1) [13] have been reported as HO inhibitors. Their chemistry and the meaningful structural insights into human HO-1 inhibition have been recently reviewed [14]. In brief, three key chemical moieties are necessary for inhibition of HO: an azolyl nucleus, a hydrophobic portion, and a central alkyl linker. This linker may have different length and may incorporate a dioxolane ring, a ketone or an alcohol function and heteroatoms such as sulfur or oxygen. Some representative compounds **1**–**7**, possessing the above-mentioned chemical features, are depicted in Fig. 1 [15–18].

Selected compounds have been more deeply studied and have shown to be effective also in intact cells and in *in vivo* models. For example, it has been demonstrated that compound **5** inhibits cell proliferation *in vitro* and tumor growth *in vivo* when tested in a model of hormone-refractory prostate cancer [19].

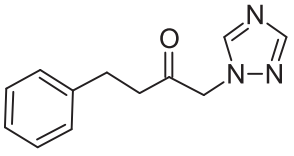
Continuing our study on imidazole-based compounds as enzymatic inhibitors [20–22], we recently investigated a series of aryloxyalkyl imidazoles as HO-1 and HO-2 inhibitors [17,18]. Among them, 1-[4-(3-bromophenoxy)butyl]-1*H*-imidazole **6** and 1-[4-(4-iodophenoxy)butyl]-1*H*-imidazole **7** (Fig. 1) showed the highest inhibitory potency. HO-1 overexpression, together with a variety of molecular mechanisms, is responsible of resistance to imatinib





azalanstat (1)

IC_{50} HO-1 = 5.5 μM, IC_{50} HO-2 = 24.5 μM



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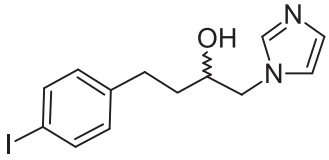
IC_{50} HO-1 = 2.6 μM, IC_{50} HO-2 = 34 μM



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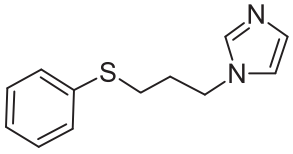
IC_{50} HO-1 = 0.06 μM, IC_{50} HO-2 = 1.8 μM



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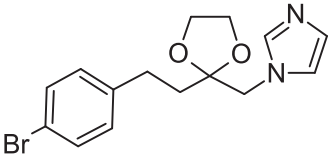
IC_{50} HO-1 = 2.4 μM, IC_{50} HO-2 = 16 μM



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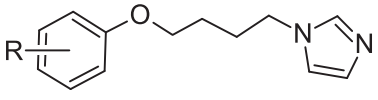
IC_{50} HO-1 = 1.9 μM, IC_{50} HO-2 = >100 μM



6, 7

6 R = 3-Br, IC_{50} HO-1 = 2.1 μM, IC_{50} HO-2 = 2.2 μM

7 R = 4-I, IC_{50} HO-1 = 1 μM, IC_{50} HO-2 = 10 μM

Fig. 1. Chemical structures and IC_{50} values of HO inhibitors **1**–**7**.

mesylate (IM) in the treatment of chronic myeloid leukemia (CML) [23–25]. In our experiments, simultaneous administration of compounds **6** and **7** with IM in CML cells overexpressing HO-1 and resistant to IM overcame resistance to IM, sensitizing these cells to the antitumor effect of IM itself and demonstrating the antitumor properties of our HO-1 inhibitors.

Based on these premises, in this work we synthesized and tested novel imidazole-based inhibitors **29**–**42** (Table 1) to examine two main chemical modifications of our lead compounds: i) the elongation of the connecting alkyl linker; ii) the replacement of the hydrophobic aryloxy moieties with bicyclic heterocyclic nuclei such as benzoxazole, benzothiazole, and 5-chlorobenzothiazole. In light of the involvement of HO system in the progress of various malignant tumors and the consequent potential usefulness of HO inhibition, we selected compounds showing the better profile as HO inhibitors, to evaluate their cytotoxic effect against prostate cancer cells (DU-145, PC3, LnCap) and breast cancer cells (MDA-MB-231, MCF-7), showing different expression pattern of HO system, and against non-cancerous canine kidney epithelial cells (MDCK).

2. Results and discussion

2.1. Chemistry

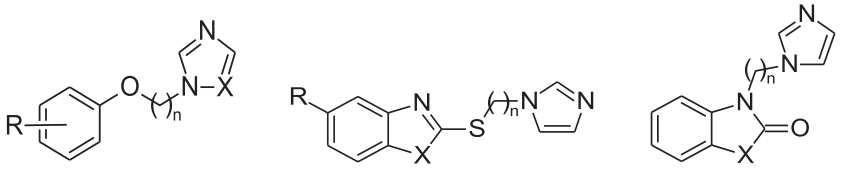
The synthesis of imidazole derivatives **29**–**42** and **50** followed the general two-steps pathways illustrated in Scheme 1. When the starting materials were phenols **8** and **9**, in the first step they were treated with appropriate dibromoalkane in DMSO, in the presence of KOH, at room temperature, to give aryloxyalkylbromides **15**–**18** in good yields. When the starting materials were 2-mercaptobenzoxazole, 2-mercaptobenzothiazole, 2-mercapto-5-chlorobenzothiazole, 2-benzoxazolone, or 2-benzothiazolone **10**–**14**, the first step was carried in acetone, at room temperature, with appropriate 1-bromo-ω-chloroalkane or dibromoalkane, in the presence of K_2CO_3. The intermediates benzoxazole, benzothiazole, or 5-chlorobenzothiazole thioalkylchlorides **19**–**26**, 2-benzoxazolone or 2-benzothiazolone alkylbromides **27** and **28** were obtained in good yields. In the second step, compounds **15**–**28** were allowed to react with imidazole or 1,2,4-triazole in acetonitrile under microwave irradiation, in the presence of triethylamine (TEA) and tetrabutylammoniumbromide (TBAB), at 90 °C for 30–45 min, to give desired final products **29**–**42** and **50** in moderate yields.

Purifications of all new compounds were performed by flash chromatography using ethyl acetate or mixtures of ethyl acetate and methanol as eluent. The structures of all new synthesized compounds were confirmed by analytical data, IR, and ^{1}H NMR spectra.

2.2. Structure–activity relationships

In this work, compounds **29**–**50** were tested to evaluate their

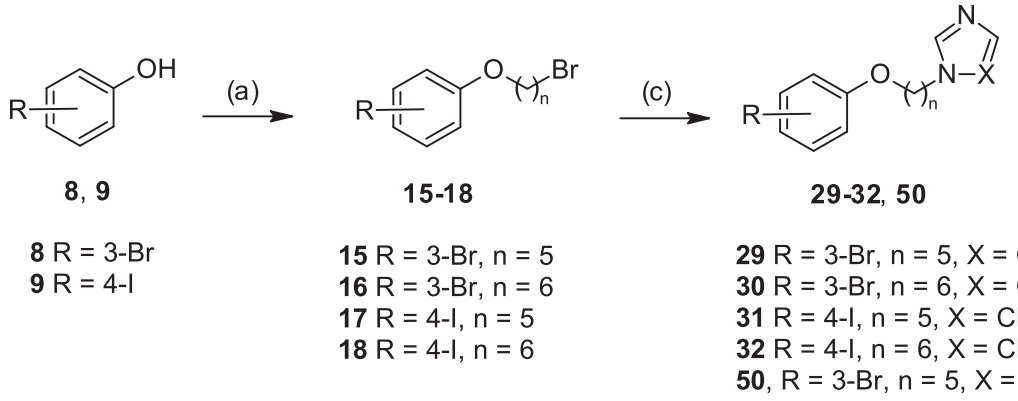
Table 1
Inhibitory potency of compounds **29**–**50** for HO-1 and HO-2.

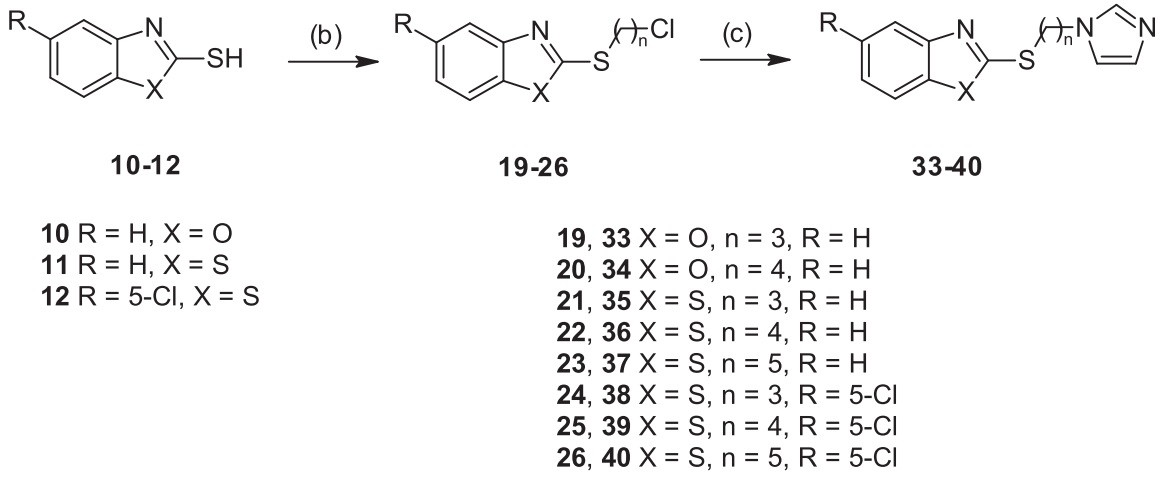


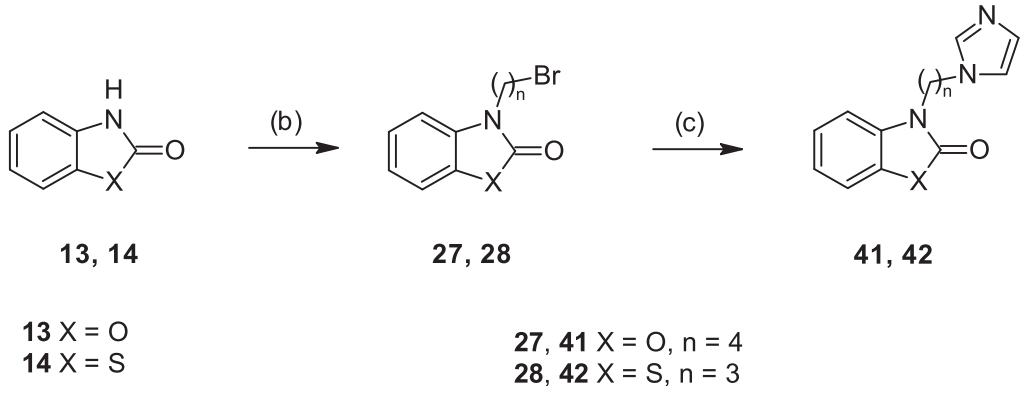
29-32, 43-50 **33-40** **41, 42**

Comp.	R	n	X	IC_{50} (μM)	
				HO-1[a]	HO-2[a]
29	3-Br	5	CH	42.6 ± 1.2	0.9 ± 0.01
30	3-Br	6	CH	34 ± 3	41 ± 3
31	4-I	5	CH	44 ± 2	0.9 ± 0.02
32	4-I	6	CH	42 ± 1	>100
33	H	3	O	16.9 ± 2.1	49 ± 4
34	H	4	O	30.9 ± 1.3	74.1 ± 5.1
35	H	3	S	0.9 ± 0.01	12.5 ± 2.5
36	H	4	S	1 ± 0.02	0.8 ± 0.01
37	H	5	S	39.8 ± 3.1	11.2 ± 2.1
38	Cl	3	S	18.4 ± 1.5	0.9 ± 0.02
39	Cl	4	S	>100	0.9 ± 0.01
40	Cl	5	S	36.3 ± 2.3	0.9 ± 0.02
41		4	O	17.7 ± 1.2	35.5 ± 1.3
42		3	S	16.6 ± 1.1	28.8 ± 3.1
43	H	3	N	>100[b]	>100
44	4-NO_2	3	CH	>100[b]	44.7 ± 4.1
45	4-NO_2	3	N	>100[b]	77.6 ± 5.2
46	4-NO_2	4	N	>100[b]	41.7 ± 3.3
47	3-Br	3	N	>100[b]	33.9 ± 2.5
48	3-Br	4	N	>100[b]	11.2 ± 0.9
49	4-I	3	CH	>100[b]	36.3 ± 2.8
50	3-Br	5	N	20 ± 1	0.8 ± 0.01
6				2.1 ± 0.3[b]	2.2 ± 0.2[b]
7				1 ± 0.01[b]	10 ± 0.5[b]

[a] Data are shown as IC_{50} values in μM ± standard deviation (SD). Values are the mean of triplicate experiments.
[b] Data taken from Ref. [18].







Scheme 1. Reagents and conditions: (a) DMSO, KOH, 1,ω-dibromoalkane, room temperature, 1 h; (b) acetone, K_2CO_3, 1-bromo-ω-chloroalkane or 1,ω-dibromoalkane, room temperature, 24 h; (c) imidazole or 1,2,4-triazole (to obtain **50**), acetonitrile, TEA, TBAB, 90 °C, MW, 30–45 min.

ability to inhibit HO-1 and HO-2 obtained from the microsomal fractions of rat spleen and rat brain, respectively. The HO-1 and HO-2 activities were determined by measuring the bilirubin formation using the difference in absorbance at 464–530 nm, as described in the experimental section. Compounds **6** and **7** (Fig. 1) were used as reference substances. Inhibition of enzyme activity is expressed as IC_{50} (μM) and results are summarized in Table 1.

In the design of novel inhibitors, we took into account previous SAR and crystallographic studies. These studies have demonstrated that the key-functional groups required for HO-1 inhibition are an imidazole nucleus and a hydrophobic moiety connected by different spacers; these last can be of different length and can incorporate various functional groups (Fig. 1). Imidazole ring is crucial for the binding to the enzyme, since it serves as an anchor by coordinating with the heme iron of the enzymatic complex; the hydrophobic moiety stabilizes the complex inhibitor-enzyme through interactions involving residues lining in hydrophobic pocket, i.e. Phe33, Met34, Phe37, Val50, Leu54, Leu147, Phe167, and Phe214. This pocket is very flexible and can accommodate various hydrophobic groups, such as phenyl, 4-chlorophenyl, adamantly, or

biphenyl moieties [15,16,26]. On these basis and taking into account **6** and **7** (Fig. 1) as lead compounds, in this work we designed, synthesized, and tested a new series of imidazole-based derivatives.

In the first part of this study, we wanted to examine the influence of the length of the linker on potency and selectivity against HO-1 or HO-2. In this regard, we synthesized and tested compounds **29**–**32**, in which the oxybutyl chain of the reference compounds **6** and **7** was replaced with oxypentyl or oxyhexyl chains. Results show that an elongation of the spacer is detrimental for HO-1 inhibition whereas it is important to improve the inhibition of HO-2. In particular, it is possible to observe a decrease of potency of the oxyhexyl derivatives **30** and **32** for both isoforms, and an increase of potency of oxypentyl derivatives **29** and **31** only for HO-2 when compared to reference compounds **6** and **7**. These results suggest that the length of the linker is crucial for the binding of these inhibitors with the active site of the enzyme.

In a second step we wanted to investigate the effect of the hydrophobic group on HO activity; with this purpose, we synthesized the imidazole derivatives **33**–**42** in which the aryl moieties of the reference compounds **6** and **7** were replaced with benzoxazole, benzothiazole, 5-chlorobenzothiazole, 2-benzoxazolone, and 2-benzothiazolone nuclei. These bicyclic heterocycles possess the chemical features required for the interaction with the hydrophobic pocket of the enzyme and some of them are part of molecules showing numerous biological activities, such as anticancer properties [27–31]. Furthermore, 2-benzoxazolone and 2-benzothiazolone are regarded as “privileged scaffolds” in medicinal chemistry [32].

Results show that benzoxazole (**33**, **34**), 2-benzoxazolone and 2-benzothiazolone (**41**, **42**) derivatives are poor HO-1 and HO-2 inhibitors, demonstrating that these heterocycles give a negative contribution to the activity. On the other hand, most interesting compounds belong to the benzothiazole series (**35**–**38**); particularly, the thiopropyl derivative **35** results a potent HO-1 inhibitor, about ten-fold selective for HO-1 over HO-2 whereas the thiobutyl derivative **36** is potent in inhibiting both isoforms. In addition, the thiopentyl derivative **37**, although less potent than its shorter homologues **35** and **36**, prefers HO-2 with respect to HO-1, confirming that a pentyl chain is crucial for the binding to HO-2, as observed in the aryloxyalkyl series **29**–**32**. Furthermore, 5-chlorobenzothiazole derivatives **38**–**40** are noteworthy since all of them are potent HO-2 inhibitors and the thiobutyl derivative **39** is the most selective HO-2 inhibitor among tested compounds.

To the best of our knowledge, very few HO-2 inhibitors, selective over HO-1, are known so far, with consequent sparse information on biological and pharmacological roles of the HO-2 isoform. Among the novel imidazole derivatives described in this work, some are very good HO-2 inhibitors selective over HO-1, particularly derivatives **29** and **31**, belonging to the aryloxyalkyl series, and compound **39**, belonging to the bicyclic series. We had previously synthesized a series of aryloxyalkyl imidazoles derivatives [18] and some of them (**43**–**49**, Table 1) were not assayed on HO-2 because they did not inhibit HO-1. In light of the interesting selective HO-2 inhibition observed for compounds **29** and **31**, we decided to test now on HO-2 the previously synthesized aryloxyalkyl imidazoles **43**–**49**. All compounds result poor inhibitors of HO-2, with the exception of 1-[4-(3-bromophenoxy)butyl]-1*H*-1,2,4-triazole **48**, with an HO-2 IC_{50} of 11.22 μM and HO-1 IC_{50} > 100 μM. This result prompted us to synthesize the pentyl homologue of **48**, i.e 1-[5-(3-bromophenoxy)pentyl]-1*H*-1,2,4-triazole **50**. This compound is a very good HO-2 inhibitor with reduced potency for HO-1. This result confirms the critical role of the pentyl spacer to determine a different binding with the two isoforms of HO. Moreover, to the best of our knowledge, compounds **29**, **31**, **39**, **48**, and **50** are, together with benzimidazole derivatives synthesized by Vlahakis et al. [33], the first potent and selective HO-2 inhibitors described so far.

2.3. In vitro cytotoxic activity

In light of the HO-1 overexpression in many tumors [34] and on the basis of the antitumor properties previously observed for compounds **6** and **7** [18], we selected compounds **6**, **7**, **31**, **35**, and **36**, showing the better profile as HO inhibitors, for further tests. Pharmacokinetics and bioavailability may affect the efficacy of a potential drug as well as pharmacodynamics. With the aim of predicting pharmacokinetic properties of the above-mentioned five compounds, we calculated with QikProp 4.3 software [35] some physicochemical and some ADME-properties. The first include logarithm of octanol–water partition coefficient (log P) and logarithm of aqueous solubility (log S), the second include membrane permeability of Caco-2 and MDCK cells (Caucasian colon adenocarcinoma and Madin–Darby canine kidney, respectively), and % human intestinal absorption (HIA). Values obtained for all these descriptors (Table 1, supporting material) indicated a good predicted cell permeation and consequent 100% HIA. On these premises, these compounds were tested to evaluate their cytotoxic activity on five cancer cell lines: prostate and breast cancer cells, both hormone therapy resistant (DU-145, PC3, and MDA-MB-231, respectively) and sensitive (LnCap and MCF-7, respectively). These cancer cells show different expression pattern of HO system. Regarding breast cancer cell lines, moderate levels of HO-1 expression could be observed in MDA-MB-231 cell line [36,37], whereas concerning to MCF7 cells, conflicting results are reported; many authors reported moderate levels of HO-1 expression [36–38] and others reported high levels of HO-1 expression in MCF7 cells [39]. Consequently, some studies show that HO-1 induction inhibits cell proliferation and invasiveness of breast cancer cells [38,40,41], other studies affirm that HO-1 may be involved in breast carcinogenesis and progression and its inhibition may contribute to reduce cell proliferation [42]. Regarding prostate cancer cell lines, overexpression of HO-1 has been reported in LnCap and DU145 cells [19], whereas expression of HO-1 was lower in PC3 than in LnCap or DU145 cells [43,44].

Cytotoxicity was determined using the sulforhodamine B colorimetric assay based on the measurement of cellular protein content. Results of cytotoxic activity of selected compounds are described in Figs. 2–4 and in Table 2. As a general comment, aryloxyalkyl imidazole derivatives **6**, **7**, and **31** result more potent than benzothiazolethioalkyl imidazole derivatives **35** and **36**; in particular, compound **31** is the most interesting compound since has a good cytotoxicity against all tumor cell lines tested.

Concerning breast cancer, all tested compounds have a good cytotoxicity in MCF7 cell line, in particular compound **31** (IC_{50} = 10.98 μM), whereas in MDA-MB-231 cell line, that express lower levels of HO-1 compared to MCF7 cells, only compounds **6** and **31** maintain a good capacity of inhibiting cell proliferation. These results might be related to the different levels of HO-1 expression in the two examined cell lines, but also might be due to the less invasiveness of hormone-responsive MCF7 with respect to MDA-MB-231 cell line.

With regard to prostate cancer, cytotoxicity of all tested compounds, with the exception of **31**, is moderate in PC3 with respect to LnCap and DU145 cell lines; this result can be related to the expression of HO-1 which is higher in LnCap and DU145 cells relative to PC3 cells. Comparing the results obtained in the two prostatic cell lines overexpressing HO-1, i.e. LnCap and DU145, it can be observed that LnCap cells are sensitive to the treatment with compounds **6**, **7**, and **31**, in particular **7** (IC_{50} = 16.93 μM), whereas

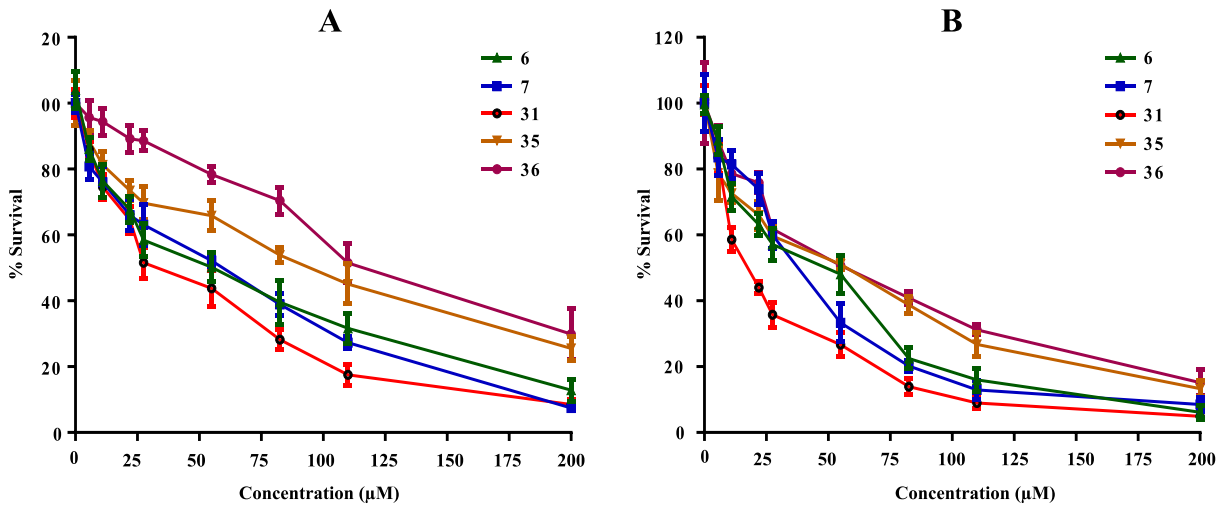


Fig. 2. Cytotoxicity of HO inhibitors against breast cancer cells. A) MDA-MB-231 (triple negative) cells and B) MCF-7 (hormone sensitive) cells were treated with 1.1–200 μM compounds **6**, **7**, **31**, **35**, and **36** for 72 h. Control wells were treated with vehicle only (0.2% DMSO). At the end of treatment Cell number was determined using the sulforhodamine B assay. Data are expressed as mean ± SEM (n = 3). Non-linear regression and IC_{50} values determination was performed using GraphPad Prism 6.

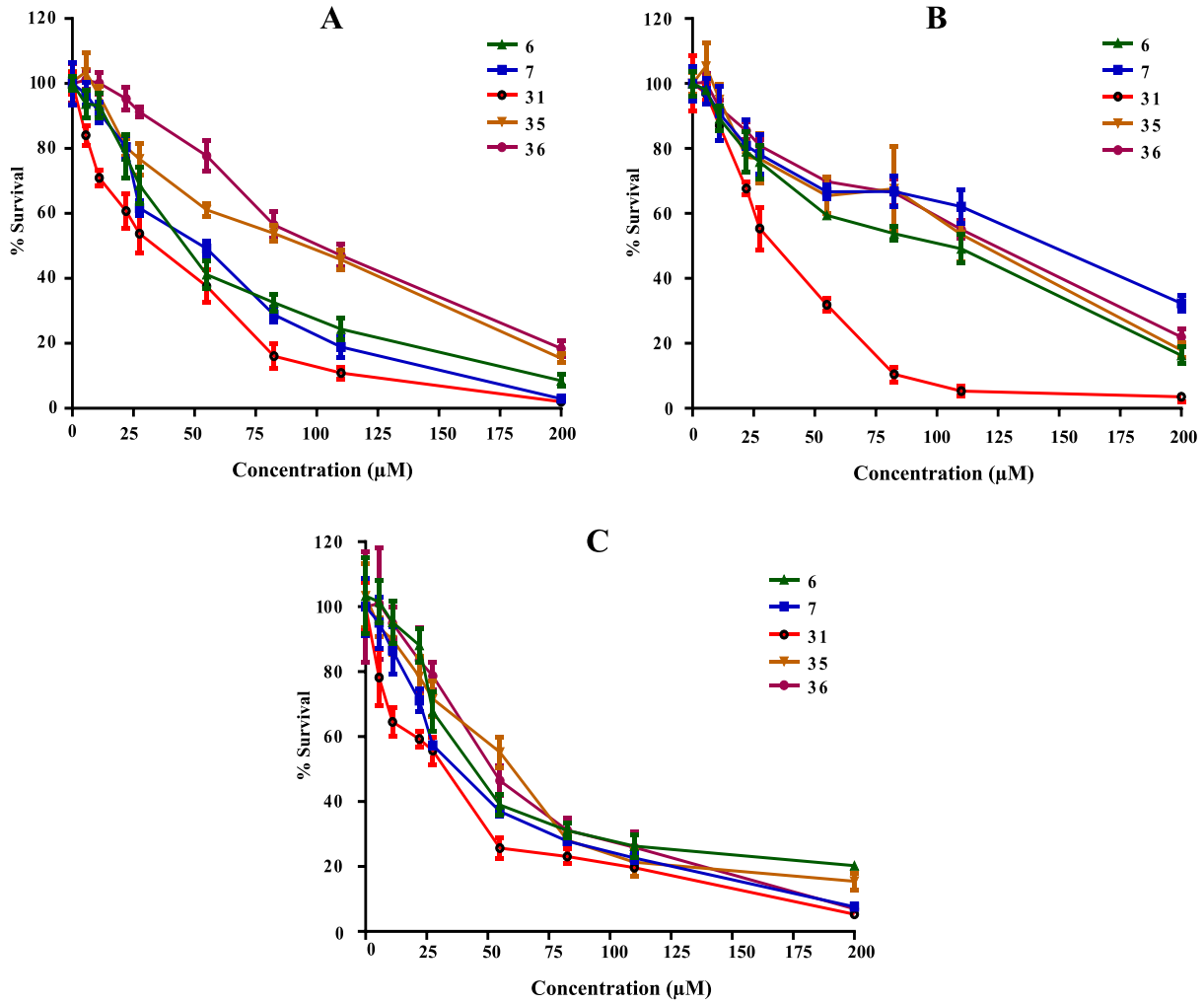


Fig. 3. Cytotoxicity of HO inhibitors against prostate cancer cells. A) DU-145 (hormone resistant) cells B) PC3 (hormone resistant) cells, and C) LnCap (hormone sensitive) cells were treated with 1.1–200 μM of compounds **6**, **7**, **31**, **35**, and **36** for 72 h. Control wells were treated with vehicle only (0.2% DMSO). At the end of treatment Cell number was determined using the sulforhodamine B assay. Data are expressed as mean ± SEM (n = 3). Non-linear regression and IC_{50} values determination was performed using GraphPad Prism 6.

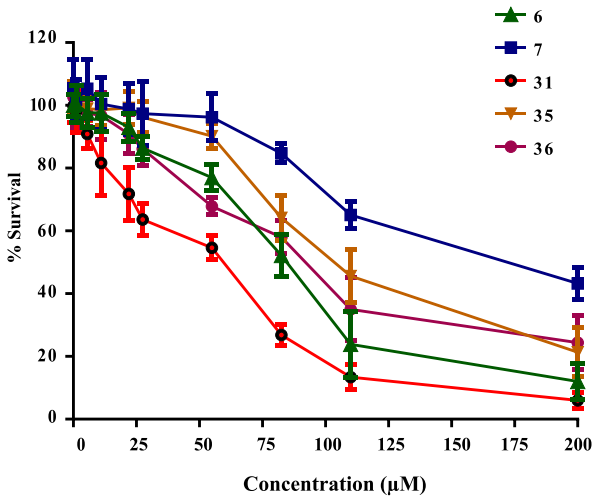


Fig. 4. Cytotoxicity of HO inhibitors against MDCK cells. MDCK (canine kidney epithelial) cells were treated with 1.1–200 μM of compound **6**, **7**, **31**, **35** and **36** for 72 h. Control wells were treated with vehicle only (0.2% DMSO). At the end of treatment cell number was determined using the sulforhodamine B assay. Data are expressed as mean ± SEM (n = 3). Non-linear regression and IC_{50} values determination was performed using GraphPad Prism 6.

DU145 cells are sensitive only to compound **31**. These effects may be due to cell type specific response. DU145 cells are considered more invasive (representative of brain metastasis) and less differentiated as compared to LnCap cells (representative of lymph node metastasis), therefore the major effect of compounds **6**, **7**, and **31** in LnCap cells respect to DU145 cells may be due to the less invasiveness of the hormone-responsive LnCap cell line.

Moreover, we the aim of getting more information on the pharmacodynamic properties of compounds **6**, **7**, **31**, **35**, and **36**, they were tested on the non-cancerous cell line MDCK, selected as example of healthy cells. All compounds are less active against MDCK cells (Fig. 4 and Table 2), resulting 2–6 fold selective for cancer cells with respect to healthy cells.

Results obtained in the present study clearly show that each cancer cell line responds differently to HO-1 or HO-2 inhibitor treatment. It is believed that intracellular localization of HO isoforms may be related to selective functions in different cell types [45,46] and several studies have implicated intracellular localization of HO-1 with different cancers [47]. On the contrary, few reports have associated HO-2 protein expression with tumor proliferation. Up to date, the role of HO-2 in cancer is almost unexplored. According to He et al. [48], HO-2 reduction may contribute to the activation of apoptotic pathway and inhibition of cell growth. Our previous results evidenced, for the first time, the ability of ellagic acid to decrease the expression of both HO-1 and HO-2 and to induce apoptosis in LnCap cells [12]. A down-regulation of the HO system and in particular of the HO-2 isoenzyme, constitutively expressed, could contribute to a significant tumor regression by decreasing HO-derived CO, mainly involved in tumor angiogenesis. At the best of our knowledge, compound **31** is the first example of HO-1 and HO-2 inhibitor more potent on HO-2 isoform, which possesses *in vitro* cytotoxic effect towards various kind of tumors.

3. Conclusions

In the present study, imidazole and triazole derivatives **29**–**50** were investigated as HO-inhibitors. Many of the tested compounds result interesting both for their potency and/or selectivity against one of the two isoforms of HO. In particular, among benzothiazole-containing molecules, thiopropyl derivative **35** is a potent inhibitor of HO-1, about ten-fold selective for HO-1 over HO-2, and the thiobutyl derivative **36** is potent toward both isoforms. Moreover, the oxypentyl derivatives **29**, **31**, **48**, **50** and the thiobutyl derivative **39**, result potent and selective inhibitors of HO-2 over HO-1, suggesting their application as pharmacological tools useful to better understand the biological roles of HO-2.

Since HO over-activity is often associated with tumor cells proliferation, compounds showing the better profile of inhibition were tested in *in vitro* models of five tumor cell lines, i.e. DU-145, PC3, LnCap, MDA-MB-231, and MCF-7, and one non-cancerous cell line, i.e. MDCK. Results of the present study show that compounds **31**, selective inhibitor of HO-2 over HO-1, is the most interesting derivative among tested compounds.

Moreover, MCF7 and LnCap cells, that represent early stage of cancer progression, are more sensitive to HO-1/HO-2 inhibition. Particularly, compound **31**, selective for HO-2, is most active in MCF7 cells expressing moderate levels of HO-1, whereas compound **7**, more potent on HO-1, is most active in LnCap cells expressing high levels of HO-1. All compounds result 2–6 fold selective for cancer cells with respect to healthy cells.

Based on these *in vitro* results, each cancer cell line responds differently to HO-1 or HO-2 inhibitor treatment, suggesting a differential expression and distinct roles of the two isoenzymes in different cancers. These findings strongly suggest that selective inhibition of HO-1 and/or HO-2 may be an option to enhance the chemotherapeutic effects of conventional anticancer drugs. In view of this consideration, it is plausible to speculate that selective inhibition of HO-1 or HO-2 in tumor cells utilizing nanotechnology, may represent a novel strategy for effective anticancer management, especially to enhance delivery to the target tumor cells without affecting normal cells.

4. Experimental procedures

4.1. Chemistry: general methods

Melting points were determined in an Electrothermal IA9200 apparatus with a digital thermometer in glass capillary tubes. Elemental analyses for C, H, N, S were within ± 0.4% of theoretical values and were performed on a Carlo Erba Elemental Analyzer

Table 2
IC_{50} values for HO inhibitor compounds in hormone resistant and hormone responsive breast, prostate cancer cells, and in non-cancerous canine kidney epithelial cells.

Comp	IC_{50} (μM)[a]					
	MDA-MB-231	MCF-7	DU145	PC3	LnCap	MDCK
6	27.46 ± 0.17	27.57 ± 0.06	46.56 ± 0.52	54.87 ± 0.53	27.61 ± 0.20	82.41 ± 0.01
7	55.06 ± 2.77	27.69 ± 0.08	54.91 ± 0.82	82.49 ± 0.61	16.93 ± 0.07	102.6 ± 0.88
31	27.44 ± 0.23	10.98 ± 0.03	27.68 ± 0.22	27.61 ± 0.21	27.79 ± 0.18	55.08 ± 0.04
35	55.12 ± 0.99	27.51 ± 0.05	54.84 ± 0.55	82.64 ± 0.98	55.03 ± 0.91	82.41 ± 0.05
36	55.07 ± 0.48	27.46 ± 0.06	82.23 ± 0.26	82.51 ± 0.12	54.73 ± 0.80	58.46 ± 3.45

[a] Data are shown as IC_{50} values in μM ± standard deviation (SD). Values are the mean of triplicate experiments.

Mod.1108 apparatus. The IR spectra were recorded in KBr disks on a Perkin Elmer 1600 series FT-IR spectrometer. ^{1}H NMR spectra were determined with a Varian Inova Unity 200 (200 MHz) instrument in DMSO-d_6 solution. Chemical shifts are in δ values (ppm) using tetramethylsilane as the internal standard; coupling constants (J) are given in Hz. Signal multiplicities are characterized as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad signal). All the synthesized compounds were checked by TLC on Merck plates (Kieselgel 60 F254) and spots were visualized under the UV light (λ = 254 and 366 nm). Preparative column chromatography was performed using Merck silica-gel 60 (230–400 mesh). Microwave irradiation experiments were carried out with a CEM Discover instrument using closed Pyrex glass tubes (ca. 10 mL) with Teflon-coated septa. All chemicals and solvents were reagent grade and were purchased from commercial sources. Analytical and spectral data of unknown compounds are following reported.

4.2. General procedure for the synthesis of aryloxyalkyl bromides **15**–**18**

Intermediates **15**–**18** were prepared following literature procedures [49]. To a DMSO solution (10 mL) was added powdered KOH (20 mmol). After stirring for 5 min, 3-bromo or 4-iodophenol was added (5 mmol), followed by appropriate 1,ω-dibromoalkanes (10 mmol). After stirring for 1 h, the reaction mixture was poured into water and extracted with CH_2Cl_2 (3 × 30 mL). The combined organic extracts were washed with brine (2 × 20 mL) and dried over anhydrous sodium sulfate. After rotary evaporation, oil residues were used without any further purification for the next step. For analytical purpose, crude **17** was purified by flash chromatography.

4.2.1. 5-(4-Iodophenoxy)pentyl bromide (**17**)

Flash chromatography on silica gel with cyclohexane/ethyl acetate (9.5:0.5, v:v) gave a white solid; mp 90.0–91.5 °C; yield 80%; IR (KBr) cm^{-1}: 2940, 1585, 1486, 1284, 1243, 1174, 817; ^{1}H NMR (DMSO-d_6) δ 7.61–7.53 (m, 2H, aromatic), 6.81–6.73 (m, 2H, aromatic), 3.93 (t, J = 6.4 Hz, 2H, OCH_2), 3.55 (t, J = 6.4 Hz, 2H, CH_2Br), 1.92–1.65 (m, 4H, $CH_2CH_2CH_2CH_2CH_2$), 1.57–1.42 (m, 2H, $CH_2CH_2CH_2$). Anal. $C_{11}H_{14}BrIO$ (C, H, N).

4.3. General procedure for the synthesis of benzoxazole, benzothiazole, or 5-chlorobenzothiazole thioalkylchlorides **19**–**26** and 2-benzoxazolone or 2-benzothiazolone alkylbromides **27**, **28**

Intermediates **19**–**26** were prepared following literature procedures, with slight modifications [50]. A solution of **10**–**14** (6.6 mmol) in acetone (20 mL) was mixed with K_2CO_3 (10 mmol) and stirred at room temperature for 10 min. After addition of the appropriate 1-bromo-ω-chloroalkane or 1,ω-dibromoalkane (7.3 mmol), the mixture was stirred for further 24 h. Inorganic materials was removed by filtration, then the solvent was removed in vacuum to obtain a residue which was used without any further purification for the next step. For analytical purpose, crude **23**–**26** were purified by flash chromatography or by recrystallization.

4.3.1. 2-[(5-Chloropentyl)thio]benzothiazole (**23**)

Flash chromatography on silica gel with cyclohexane/ethyl acetate (9:1, v:v) gave an orange oil; yield 75%; IR (KBr) cm^{-1}: 3061, 2937, 1456, 1427, 1309, 1239, 995, 756. ^{1}H NMR (DMSO-d_6) δ 8.03–7.98 (m, 1H, aromatic), 7.88–7.83 (m, 1H, aromatic), 7.05–7.32 (m, 2H, aromatic), 3.64 (t, J = 6.6 Hz, 2H, CH_2Cl), 3.38 (t, J = 7.0 Hz, 2H, SCH_2), 1.87–1.67 (m, 4H, $CH_2CH_2CH_2CH_2CH_2$), 1.62–1.46 (m, 2H, $CH_2CH_2CH_2$). Anal.$C_{12}H_{14}ClNS_2$ (C, H, N, S).

4.3.2. 2-[(3-Chloropropyl)thio]-5-chlorobenzothiazole (**24**)

Flash chromatography on silica gel with cyclohexane/ethyl acetate (9.8:0.2, v:v) gave a white solid; mp 50.0–51.0 °C; yield 72%; IR (KBr) cm^{-1}: 2961, 1584, 1546, 1453, 1425, 1247, 1082; ^{1}H NMR (DMSO-d_6) δ 8.08–8.04 (m, 1H, aromatic), 7.95–7.94 (m, 1H, aromatic), 7.45–7.40 (m, 1H, aromatic), 3.79 (t, J = 6.4 Hz, 2H, CH_2Cl), 3.48 (t, J = 6.8 Hz, 2H, SCH_2), 2.30–2.17 (m, 2H, $CH_2CH_2CH_2$). Anal. $C_{10}H_9Cl_2NS_2$ (C, H, N, S).

4.3.3. 2-[(4-Chlorobutyl)thio]-5-chlorobenzothiazole (**25**)

Recrystallization by petroleum ether 40–60 °C gave a white solid; mp 49.0–50.0 °C; yield 69%; IR (KBr) cm^{-1}: 3089, 2953, 1544, 1425, 1321, 1007, 812; ^{1}H NMR (DMSO-d_6) δ 8.07–8.03 (m, 1H, aromatic), 7.94–7.93 (m, 1H, aromatic), 7.45–7.39 (m, 1H, aromatic), 3.72 (t, J = 6.2 Hz, 2H, CH_2Cl), 3.41 (t, J = 6.8 Hz, 2H, SCH_2), 1.95–1.86 (m, 4H, $CH_2CH_2CH_2CH_2$). Anal. $C_{11}H_{11}Cl_2NS_2$ (C, H, N, S).

4.3.4. 2-[(5-Chloropentyl)thio]-5-chlorobenzothiazole (**26**)

Flash chromatography on silica gel with cyclohexane/ethyl acetate (9.8:0.2, v:v) gave an orange oil; yield 85%; IR (KBr) cm^{-1}: 3066, 2937, 1547, 1455, 1428, 1296, 1067, 996; ^{1}H NMR (DMSO-d_6) δ 8.07–8.03 (m, 1H, aromatic), 7.93–7.92 (m, 1H, aromatic), 7.45–7.39 (m, 1H, aromatic), 3.65 (t, J = 6.6 Hz, 2H, CH_2Cl), 3.38 (t, J = 7.2 Hz, 2H, SCH_2), 1.90–1.68 (m, 4H, $CH_2CH_2CH_2CH_2CH_2$), 1.62–1.46 (m, 2H, $CH_2CH_2CH_2$). Anal. $C_{12}H_{13}Cl_2NS_2$ (C, H, N, S).

4.4. General procedure for the synthesis of imidazole derivatives **29**–**42**, **50**

A literature method was employed, with modifications [51]. To a suspension of the appropriate intermediates **15**–**28** (5 mmol) in acetonitrile (3 mL), imidazole or 1,2,4 triazole (7.5 mmol), TEA (5 mmol), and a catalytic amount of TBAB (0.1 g) were added. The mixture and a magnetic bar was sealed in a Pyrex test tube (ca. 10 mL) and heated at 90 °C under microwaves irradiation for 30–45 min (run time 2 min, microwave max power 150 W, max pressure 150 Psi). The solvent was eliminated in vacuo to give a residue which was suspended in water; the mixture was alkalinized with NaOH 0.1 N and extracted with ethyl acetate (3 × 50 mL). The combined extracts were washed with water, dried on anhydrous sodium sulfate, and evaporated to obtain a residue which was purified by flash column chromatography on silica gel using ethyl acetate or mixtures of ethyl acetate/methanol as eluent.

By use of this procedure, the subsequent new compounds were obtained:

4.4.1. 1-[5-(3-Bromophenoxy)pentyl]-1H-imidazole (**29**)

Flash chromatography on silica gel with ethyl acetate/methanol (9:1, v:v) gave a colorless oil; yield 18%; IR (KBr) cm^{-1}: 3108, 2940, 2868, 1588, 1508, 1468, 1294, 1228,1077; ^{1}H NMR (DMSO-d_6) δ 7.61 (s, 1H, imidazole), 7.27–7.08 (m, 3H aromatic + 1H imidazole), 6.95–6.87 (m, 1H aromatic + 1H imidazole), 4.00–9.93 (m, 4H, OCH_2 + CH_2N), 1.83–1.64 (m, 4H, $CH_2CH_2CH_2CH_2CH_2$), 1.40–1.28 (m, 2H, $CH_2CH_2CH_2CH_2CH_2$). Anal. $C_{14}H_{17}BrN_2O$ (C, H, N).

4.4.2. 1-[6-(3-Bromophenoxy)hexyl]-1H-imidazole (**30**)

Flash chromatography on silica gel with ethyl acetate/methanol (9:1, v:v) gave a light yellow oil; yield 19%; IR (KBr) cm^{-1}: 3107, 2937, 2860, 1588, 1572, 1486, 1284, 1228, 1077; ^{1}H NMR (DMSO-d_6) δ 7.60 (s, 1H, imidazole), 7.27–7.08 (m, 3H aromatic + 1H imidazole), 6.95–6.87 (m, 1H aromatic +1H imidazole), 3.98–3.91 (m, 4H, OCH_2 + CH_2N), 1.75–1.61 (m, 4H, $CH_2CH_2CH_2CH_2CH_2CH_2$), 1.48–1.24 (m, 4H, $CH_2CH_2CH_2CH_2CH_2CH_2$). Anal. $C_{15}H_{19}BrN_2O$ (C, H, N).

*4.4.3. 1-[5-(4-Iodophenoxy)pentyl]-1H-imidazole (**31**)*

Flash chromatography on silica gel with ethyl acetate/methanol (9.5:0.5, v:v) gave a white solid; mp 90.9–92.9 °C; yield 15%; IR (KBr) cm^{-1}: 3096, 2933, 2861, 1585, 1510, 1487, 1286, 1234, 1176, 1076; ^1H NMR (DMSO-d_6) δ 7.61–7.52 (m, 2H aromatic + 1H imidazole), 7.15 (br s, 1H, imidazole), 6.86 (br s, 1H, imidazole), 6.79–6.72 (m, 2H aromatic), 3.99–3.88 (m, 4H, OCH_2 + CH_2N), 1.78–1.63 (m, 4H, $CH_2CH_2CH_2CH_2CH_2$), 1.39–1.27 (m, 2H, $CH_2CH_2CH_2CH_2CH_2$). Anal. $C_{14}H_{17}IN_2O$ (C, H, N).

*4.4.4. 1-[6-(4-Iodophenoxy)hexyl]-1H-imidazole (**32**)*

Flash chromatography on silica gel with ethyl acetate/methanol (9:1, v:v) gave a white solid; mp 85.6–87.3 °C; yield 21%; IR (KBr) cm^{-1}: 3103, 2940, 2855, 1586, 1488, 1284, 1244, 1101, 811; ^1H NMR (DMSO-d_6) δ 7.60–7.54 (m, 2H aromatic + 1H imidazole), 7.15 (br s, 1H, imidazole), 6.86 (br s, 1H, imidazole), 6.80–6.72 (m, 2H aromatic), 3.97–3.87 (m, 4H, OCH_2 + CH_2N), 1.78–1.60 (m, 4H, $CH_2CH_2CH_2CH_2CH_2CH_2$), 1.47–1.21 (m, 4H, $CH_2CH_2CH_2CH_2CH_2CH_2$). Anal. $C_{15}H_{19}IN_2O$ (C, H, N).

*4.4.5. 2-[[3-(1H-Imidazol-1-yl)propyl]thio]benzoxazole (**33**)*

Flash chromatography on silica gel with ethyl acetate/methanol (9.5:0.5, v:v) gave a yellow oil; yield 22%; IR (KBr) cm^{-1}: 3108, 2938, 1600, 1501, 1453, 1237, 1131, 1096, 744; ^1H NMR (DMSO-d_6) δ 7.68–7.60 (m, 2H aromatic + 1H imidazole), 7.38–7.29 (m, 2H aromatic), 7.23 (br s, 1H, imidazole), 6.91 (br s, 1H, imidazole), 4.13 (t, J = 6.8 Hz, 2H, CH_2N), 3.26 (t, J = 7.2 Hz, 2H, SCH_2), 2.32–2.18 (m, 2H, $CH_2CH_2CH_2$). Anal. $C_{13}H_{13}N_3OS$ (C, H, N, S).

*4.4.6. 2-[[4-(1H-Imidazol-1-yl)butyl]thio]benzoxazole (**34**)*

Flash chromatography on silica gel with ethyl acetate/methanol (9:1, v:v) gave a colorless oil; yield 20%; IR (KBr) cm^{-1}: 3109, 2940, 1499, 1453 1237, 1131, 1096, 744; ^1H NMR (DMSO-d_6) δ 7.68–7.59 (m, 2H aromatic + 1H imidazole), 7.37–7.26 (m, 2H aromatic), 7.17 (br s, 1H, imidazole), 6.86 (br s, 1H, imidazole), 4.01 (t, J = 6.8 Hz, 2H, CH_2N), 3.39 (t, J = 7.2 Hz, 2H, SCH_2), 1.90–1.67 (m, 4H, $CH_2CH_2CH_2CH_2$). Anal. $C_{14}H_{15}N_3OS$ (C, H, N, S).

*4.4.7. 2-[[3-(1H-Imidazol-1-yl)propyl]thio]benzothiazole (**35**)*

Flash chromatography on silica gel with ethyl acetate/methanol (9:1, v:v) gave a light brown oil; yield 23%; IR (KBr) cm^{-1}: 3109, 2935, 1507, 1456, 1427, 1232, 1078, 997, 757; ^1H NMR (DMSO-d_6) δ 8.03–7.99 (m, 1H aromatic), 7.98–7.93 (m, 1H aromatic), 7.68 (s, 1H, imidazole), 7.51–7.32 (m, 2H, aromatic), 7.23 (br s, 1H, imidazole), 6.92 (br s, 1H, imidazole), 4.13 (t, J = 6.8 Hz, 2H, CH_2N), 3.29 (t, J = 7.2 Hz, 2H, SCH_2), 2.30–2.16 (m, 2H, $CH_2CH_2CH_2$). Anal. $C_{13}H_{13}N_3S_2$ (C, H, N, S).

*4.4.8. 2-[[4-(1H-Imidazol-1-yl)butyl]thio]benzothiazole (**36**)*

Flash chromatography on silica gel with ethyl acetate/methanol (9:1, v:v) gave a light yellow solid; mp 85.0–86.0 °C; yield 27%; IR (KBr) cm^{-1}: 3096, 2946, 1511, 1453, 1369, 1225, 1081, 1005, 754; ^1H NMR (DMSO-d_6) δ 8.02–7.98 (m, 1H aromatic), 7.87–7.83 (m, 1H aromatic), 7.63 (s, 1H, imidazole), 7.50–7.32 (m, 2H, aromatic), 7.17 (br s, 1H, imidazole), 6.86 (br s, 1H, imidazole), 4.01 (t, J = 6.8 Hz, 2H, CH_2N), 3.37 (t, J = 7.2 Hz, 2H, SCH_2), 1.90–1.66 (m, 4H, $CH_2CH_2CH_2CH_2$). Anal. $C_{14}H_{15}N_3S_2$ (C, H, N, S).

*4.4.9. 2-[[5-(1H-Imidazol-1-yl)pentyl]thio]benzothiazole (**37**)*

Flash chromatography on silica gel with ethyl acetate/methanol (9:1, v:v) gave a pale orange oil; yield 20%; IR (KBr) cm^{-1}: 3107, 2935, 1507, 1456, 1426, 1309, 1232, 996, 758; ^1H NMR (DMSO-d_6) δ 8.02–7.98 (m, 1H aromatic), 7.86–7.82 (m, 1H aromatic), 7.60 (s, 1H, imidazole), 7.50–7.31 (m, 2H, aromatic), 7.15 (br s, 1H, imidazole), 6.86 (br s, 1H, imidazole), 3.95 (t, J = 7.0 Hz, 2H, CH_2N), 3.37 (t, J = 7.2 Hz, 2H, SCH_2), 1.85–1.67 (m, 4H, $CH_2CH_2CH_2CH_2CH_2$), 1.43–1.31 (m, 2H, $CH_2CH_2CH_2CH_2CH_2$). Anal. $C_{15}H_{17}N_3S_2$ (C, H, N, S).

*4.4.10. 2-[[3-(1H-Imidazol-1-yl]propyl]thio]-5-chlorobenzothiazole (**38**)*

Flash chromatography on silica gel with ethyl acetate/methanol (9:1, v:v) gave a light orange oil; yield 23%; IR (KBr) cm^{-1}: 3108, 2936, 1507, 1455, 1428, 1230, 1078, 1003, 908; ^1H NMR (DMSO-d_6) δ 8.07–8.02 (m, 1H aromatic), 7.92–7.91 (m, 1H aromatic), 7.67 (s, 1H, imidazole), 7.45–7.39 (m, 1H, aromatic), 7.23 (s, 1H, imidazole), 6.91 (s, 1H, imidazole), 4.13 (t, J = 7.0 Hz, 2H, CH_2N), 3.25 (t, J = 7.6 Hz, 2H, SCH_2), 2.30–2.16 (m, 2H, $CH_2CH_2CH_2$). Anal. $C_{13}H_{12}ClN_3S_2$ (C, H, N, S).

*4.4.11. 2-[[4-(1H-Imidazol-1-yl)butyl]thio]-5-chlorobenzothiazole (**39**)*

Flash chromatography on silica gel with ethyl acetate/methanol (9.5:0.5, v:v) gave a light yellow solid; mp 72.4–73.1 °C; yield 21%; IR (KBr) cm^{-1}: 3090, 2838, 1546, 1508, 1427, 1289, 1240, 1070, 804; ^1H NMR (DMSO-d_6) δ 8.06–8.02 (m, 1H aromatic), 7.94–7.93 (m, 1H aromatic), 7.65 (br s, 1H, imidazole), 7.44–7.39 (m, 1H, aromatic), 7.18 (br s, 1H, imidazole), 6.88 (br s, 1H, imidazole), 4.02 (t, J = 6.6 Hz, 2H, CH_2N), 3.37 (t, J = 7.4 Hz, 2H, SCH_2), 1.90–1.63 (m, 4H, $CH_2CH_2CH_2CH_2$). Anal. $C_{14}H_{14}ClN_3S_2$ (C, H, N, S).

*4.4.12. 2-[[5-(1H-Imidazol-1-yl)pentyl]thio]-5-chlorobenzothiazole (**40**)*

Flash chromatography on silica gel with ethyl acetate gave a colorless oil; yield 17%; IR (KBr) cm^{-1}: 3107, 2935, 2859, 1507, 1454, 1427, 1229, 1068, 1001; ^1H NMR (DMSO-d_6) δ 8.07–8.02 (m, 1H aromatic), 7.93–7.92 (m, 1H aromatic), 7.61 (s, 1H, imidazole), 7.44–7.39 (m, 1H, aromatic), 7.15 (s, 1H, imidazole), 6.87 (s, 1H, imidazole), 3.96 (t, J = 7.0 Hz, 2H, CH_2N), 3.35 (t, J = 7.6 Hz, 2H, SCH_2), 1.86–1.67 (m, 4H, $CH_2CH_2CH_2CH_2CH_2$), 1.42–1.31 (m, 2H, $CH_2CH_2CH_2$). Anal. $C_{15}H_{16}ClN_3S_2$ (C, H, N, S).

*4.4.13. 3-[4-(1H-Imidazol-1-yl)butyl]-2(3H)-benzoxazolone (**41**)*

Flash chromatography on silica gel with ethyl acetate/methanol (9:1, v:v) gave a white solid; mp 90.1–91.9 °C; yield 20%; IR (KBr) cm^{-1}: 3133, 2943, 1767, 1611, 1498, 1351, 1247, 1078, 755; ^1H NMR (DMSO-d_6) δ 7.60 (s, 1H, aromatic), 7.36–7.08 (m, 4H aromatic + 1H imidazole), 6.86 (br s, 1H, imidazole), 3.98 (t, J = 6.6 Hz, 2H, CH_2N) 3.82 (t, J = 7.0 Hz, 2H, $CONCH_2$), 1.78–1.56 (m, 4H, $CH_2CH_2CH_2CH_2$). Anal. $C_{14}H_{15}N_3O_2$ (C, H, N).

*4.4.14. 3-[3-(1H-Imidazol-1-yl)propyl]-2(3H)-benzothiazolone (**42**)*

Flash chromatography on silica gel with ethyl acetate/methanol (9:1, v:v) gave a thick colorless oil; yield 24%; IR (KBr) cm^{-1}: 3110, 2944, 1669, 1588, 1509, 1472, 1327, 1230, 749; ^1H NMR (DMSO-d_6) δ 7.60 (s, 1H, aromatic), 7.36–7.08 (m, 4H aromatic + 1H imidazole), 6.86 (br s, 1H, imidazole), 3.98 (t, J = 6.6 Hz, 2H, CH_2N) 3.82 (t, J = 7.0 Hz, 2H, $CONCH_2$), 1.78–1.56 (m, 2H, $CH_2CH_2CH_2$). Anal. $C_{13}H_{13}N_3OS$ (C, H, N, S).

*4.4.15. 1-[5-(3-Bromophenoxy)pentyl]-1H-1,2,4-triazole (**50**)*

Flash chromatography on silica gel with ethyl acetate gave a colorless oil; yield 25%; IR (KBr) cm^{-1}: 3118, 2944, 2869, 1589, 1572, 1469, 1273, 1228, 1014; ^1H NMR (DMSO-d_6) δ 8.41 (s, 1H, triazole), 7.95 (s, 1H, triazole), 7.26–7.18 (m, 1H aromatic), 7.12–7.07 (m, 2H, aromatic), 6.96–6.88 (m, 1H, aromatic), 4.19 (t, J = 6.8 Hz, 2H, OCH_2), 3.95 (t, J = 6.4 Hz, 2H, CH_2N), 1.86–1.64 (m, 4H, $CH_2CH_2CH_2CH_2CH_2$), 1.40–1.28 (m, 2H, $CH_2CH_2CH_2CH_2CH_2$). Anal. $C_{13}H_{16}BrN_3O$ (C, H, N).

4.5. Biology

4.5.1. Preparation of spleen and brain microsomal fractions

HO-1 and HO-2 were obtained, respectively, from rat spleen and brain as the microsomal fraction prepared by differential centrifugation; the dominance of HO-1 protein in the rat spleen and of HO-2 in the rat brain has been well documented [33]. These particular microsomal preparations were selected in order to use the most native (i.e., closest to *in vivo*) forms of HO-1 and HO-2. Spleen and brain (Sprague–Dawley rats) microsomal fractions were prepared according to the procedure outlined by Ryter et al. [4]. The experiments reported in the present paper complied with current Italian law and met the guidelines of the Institutional Animal Care and Use Committee of University of Catania (Italy). The experiments were performed in male Sprague–Dawley albino rats (150 g body weight and age 45 d). They had free access to water and were kept at room temperature with a natural photo-period (12 h light-12 h dark cycle). For measuring HO-1 and HO-2 activities, each rat was sacrificed and their spleen and brain were excised and weighed. A homogenate (15%, w/v) of spleens and brains pooled from four rats was prepared in ice-cold HO-homogenizing buffer (50 mM Tris buffer, pH 7.4, containing 0.25 M sucrose) using a Potter–Elvehjem homogenizing system with a Teflon pestle. The microsomal fraction of rat spleen and brain homogenate was obtained by centrifugation at 10,000 g for 20 min at 4 °C, followed by centrifugation of the supernatant at 100,000 g for 60 min at 4 °C. The 100,000 g pellet (microsomes) was resuspended in 100 mM potassium phosphate buffer, pH 7.8, containing 2 mM $MgCl_2$ with a Potter–Elvehjem homogenizing system. The rat spleen and brain microsomal fractions were divided into equal aliquots, placed into microcentrifuge tubes, and stored at −80 °C for up to 2 months. Protein concentration of the microsomal fraction was determined by Lowry method [52].

4.5.2. Preparation of biliverdin reductase

Liver cytosol has been used as a source of biliverdin reductase. Rat liver was perfused through the hepatic portal vein with cold 0.9% NaCl, then it was cut and flushed with 2 × 20 mL of ice cold PBS to remove all of the blood. Liver tissue was homogenized in 3 volumes of solution containing 1.15% KCl w/v and Tris buffer 20 mM, pH 7.8 on ice. Homogenates were centrifuged at 10,000 *g*, for 20 min at 4 °C. Supernatant was decanted and centrifuged at 100,000 *g* for 1 h at 4 °C to sediment the microsomes. The 100,000 *g* supernatant was saved and then stored in small amounts at −80 °C after its protein concentration was measured.

4.5.3. Measurement of HO-1 and HO-2 enzymatic activities in microsomal fraction of rat spleen and brain

The HO-1 and HO-2 activities were determined by measuring the bilirubin formation using the difference in absorbance at 464–530 nm as described by Ryter et al. [4]. Reaction mixtures (500 μL) consisted of 20 mM Tris–HCl, pH 7.4, (1 mg/mL) microsomal extract, 0.5–2 mg/mL biliverdin reductase, 1 mM NADPH, 2 mM glucose 6-phosphate (G6P), 1 U G6P dehydrogenase, 25 μM hemin, and 10 μL of DMSO (or the same volume of DMSO solution of test compounds to a final concentration of 100, 10, and 1 μM). Incubations were carried out for 60 min at 37 °C in a circulating water bath in the dark. Reactions were stopped by adding the same volume of chloroform. After recovering the chloroform phase, the amount of bilirubin formed was measured with a double-beam spectrophotometer as $OD_{464-530}$ nm (extinction coefficient, 40 mM/cm^{-1} for bilirubin). One unit of the enzyme was defined as the amount of enzyme catalyzing the formation of 1 nmol of bilirubin/mg protein/h.

4.5.4. Cell cultures

MDA-MB-231 cells (hormone resistant), MCF-7 (hormone sensitive), PC3 cells (hormone resistant), DU145 cells (hormone resistant), LnCap cells (hormone sensitive), and MDCK (non-cancerous canine kidney epithelial cells), were maintained in complete growth media (DMEM/Ham's F12 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, 100 units/mL of streptomycin, and 2.2 g/L of $NaHCO_3$) at 37 °C in a humidified atmosphere of 5% CO_2. For all procedures, cells were harvested using TrypLE Express (Life Technologies, Auckland, New Zealand).

4.5.5. In vitro cytotoxicity of HO inhibitors against breast and prostate cancer cell lines and against non-cancerous kidney epithelial cells

To test the cytotoxic effect of HO inhibitors, cells were seeded into 96-well plates and incubated for 24 h. This was followed by treatment with HO inhibitors at the concentrations indicated for 72 h. Following the incubation, cells were fixed using 10% trichloroacetic acid (TCA). Cytotoxicity was determined using the sulforhodamine B assay as previously described [53]. The concentration required to decrease the cell number by 50% (IC_{50}) was determined by non-linear regression using Graphpad prism 6 software. Treatments were performed in triplicate and data represents mean of three independent experiments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.04.003.

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