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Evaluation of novel aryloxyalkyl derivatives of imidazole and 1,2,4-triazole as heme oxygenase-1 (HO-1) inhibitors and their antitumor properties

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ABSTRACT

A novel series of aryloxyalkyl derivatives of imidazole and 1,2,4-triazole, **17**–**31**, was designed and synthesized as inhibitors of heme oxygenase-1 (HO-1) and heme oxygenase-2 (HO-2). Some of these compounds were found to be good inhibitors of HO-1, in particular those carrying an imidazole moiety as azolyl group and a 3-bromo or 4-iodophenyl as aryl moiety. The most potent compounds **6** and **30** were selected and studied for their antitumor properties in a model of LAMA-84 R cell line overexpressing HO-1 and resistant to imatinib mesylate (IM), a tyrosine-kinase inhibitor used in the treatment of multiple types of cancer, most notably Philadelphia Chromosome positive (Ph^+) Chronic Myelogenous Leukemia (CML). Results show that both **6** and **30** sensitized LAMA-84 R cell line to antitumor properties of IM.

1. Introduction

Heme oxygenase (HO) is the enzyme which catalyzes the regioselective, oxidative degradation of heme, with the simultaneous release of carbon monoxide (CO), ferrous iron (Fe^{2+}), and biliverdin, this last further reduced to bilirubin by biliverdin reductase. To date three enzyme isoforms have been identified: HO-1, HO-2, and HO-3. HO-1, the most studied isoform also known as heat shock protein 32 (Hsp32), is an inducible 32-kDa enzyme, predominantly expressed in the liver and spleen, that can be stress-induced by a variety of stimuli such as heavy metals, reactive oxygen species and, particularly, heat shock. HO-2 is a constitutively expressed 36-kDa protein, widely distributed, with its high levels in the brain, testes, or endothelial cells. HO-3 is regarded as a pseudogene of HO-2 and its functional activity is still uncertain.[1]

The main physiological role of the HO system is protection of cells against oxidative stress.[2] This effect may be attributed both to the degradation of the free heme (which is a pro-oxidant agent since it can catalyze the production of free radicals through Fenton chemistry) and to the complementary contribution of each of the metabolites produced. In fact, CO and biliverdin, originally viewed as waste products, have been recently and widely recognized for their positive cellular regulatory actions. In particular, CO is involved as a gasotransmitter in antiinflammatory, antiapoptotic, antiproliferative and vasodilator effects,[3] biliverdin and bilirubin possess antioxidant properties.[4]

For these reasons, the HO system has generally demonstrated protective roles towards a variety of stress-induced pathology, such as diabetes, obesity, atherosclerosis, inflammation, vascular injury, transplantation, ocular diseases, hypoxia and ischemia;[5] consequently, in these pathological conditions an up-regulation of HO-1 may have therapeutic potential.[6,7] However, an up-regulation of the HO system is a double-edge sword and may not exert exclusively cytoprotective effects, but may contribute to tissue injury under certain pathologic states in which, conversely, inhibition of HO activity may have therapeutic use.[8] A growing body of evidence suggests that an up-regulation of the HO-1 along with its catabolism products play a role in the formation, growth, and metastasis of different tumors including prostate tumor,[9] Chronic Myelogenous Leukemia (CML),[10] human renal cell carcinoma,[11] and lymphosarcomas.[12] It has been reported that in human gliomas and melanomas, HO-1 is linked to angiogenesis[13,14] and, in an experimental mouse model, HO-1 accelerates pancreatic cancer growth by promoting tumor angiogenesis.[15]

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In addition, it has been found that expression of HO-1 is increased in response to several anticancer treatments, and, by the means of the protective role of HO-1 in tumor cells, could be responsible of the development of drug-resistance.[16–20] These results support the idea that HO-1 (particularly its inhibition or down-regulation), may be a good target in antitumor therapy. The potential role of HO-1 inhibitors in antitumor therapy has been demonstrated by the use of the competitive inhibitors metalloporphyrins (tin/zinc/chromium protoporphyrins), Mps, in the treatment of some tumors. Mps are heme derivatives in which the central iron atom of heme is replaced by another element, that compete with heme for binding to the enzyme. For example, administration of tin protoporphyrin (SnPP) accentuated the clinical response to 5-aminolevulinic acid-based photodynamic therapy in cultured melanoma tissue[21] and had a negative effect on growth of Kaposi sarcomas.[22] Zinc protoporphyrin IX (ZnPPIX) significantly suppressed the growth of lung cancer cell lines[23] and augmented the response to radiotherapy in nasopharyngeal carcinomas.[24]

To date, the main potential therapeutic application of HO-1 inhibitors belonging to the class of Mps, is the treatment of neonatal hyperbilirubinemia, as confirmed by a number of animal and clinical studies.[25–27] However, MPs still have not left the clinical study stage for their actual applications in human neonates, due to some limitations in their use, mainly the photosensitizing action of these compounds.[28] Some clinical studies demonstrated that using a special light with a narrow spectrum (maximum intensity 440–460 nm), photosensitivity of SnMP was a non-issue.[29] Nevertheless, since the phototoxicity of MPs appears to be strongly dependent on the irradiation and spectral quality of the light source,[28–30] the potential use of MPs in diseases different from hyperbilirubinemia, such as tumors, could be precluded.

The non-selectivity towards HO-1 and HO-2 isoforms[31] and other heme-utilizing enzymes, such as soluble guanylyl cyclase (sGC), nitric oxide synthase (NOS), and cytochrome P450 (CYP450),[32–34] may represent another problem in the use of MPs. In fact, although studies have demonstrated that some of MPs maintain selectivity against HO in vitro if used within a defined concentration range,[33] therapeutic window is narrow and has not been yet well defined; in addition antitumor activity is reported only at very high concentrations that also cause inhibition of other heme-containing enzymes.[35]

Finally the most of Mps inhibits heme oxygenase activity but may be also very potent HO-1 inducers in vivo.[36,37] Consequently, in the last few years, in the medicinal chemistry field there has been an increasing interest in the development of HO-1 inhibitors structurally distinct from the heme-analogues Mps.

The first discovered non-porphyrin HO-1 inhibitor which acts in a non-competitive manner with respect to heme, is azalanstat (**1**, Fig. 1).[38] Starting from the structure of this imidazole-containing molecule, a number of azole-based compounds has been described in literature as HO inhibitors. Their chemistry and the meaningful structural insights into human HO-1 inhibition have been recently reviewed.[39,40] Some representative azole-based HO-1 inhibitors (**2–4**) are depicted in Figure 1. On the basis of the results of both X-ray crystallography studies [41,42] and traditional structure–activity relationship (SAR) studies,[43–46] it is possible to affirm that the key functional groups which are necessary for enzyme interaction include an azolyl moiety, a hydrophobic aryl group, and a connecting alkyl chain, usually constituted by four carbons, which incorporates a dioxolanyl, a hydroxyl or a carbonyl group in the most interesting substances. Recently, the effect of the introduction of heteroatoms in the alkyl linker was investigated by compounds of general formula **5** (Fig. 1);[47] interesting results were obtained with compounds bearing a four or five carbon linker incorporating oxygen or sulfur as heteroatoms.

Our research group has recently studied a small class of imidazole-based derivatives behaving as HO-1 inhibitors,[48] selected from previous extensive research conducted in the field of NOS inhibitors.[49,50] Among them, 1-[4-(3-bromophenoxy)butyl]-1*H*-imidazole (**6**) emerged for its potency (Fig. 1). In this work, a novel series of aryloxyalkyl derivatives of imidazole and 1,2,4-triazole (**17–31**) was designed, synthesized and tested on HO-1 and HO-2 enzymes obtained from the microsomal fractions of rat spleen and rat brain, respectively. Taking into account **6** as lead-compound, we evaluated how the variation of the azolyl moiety, the introduction of different substituents on phenyl ring and the modification of the length of the connecting oxyalkyl chain, can influence HO activity.

Moreover, in light of the involvement of HO system in carcinogenesis, to further establish the utility of HO-1 inhibitors in antitumor therapy it is necessary to explore their pharmacology in experimental conditions other than broken-cell preparations, such as the above-mentioned microsomal fractions. Ideally, the range should span from intact cells to whole organs and animals in vivo. Herein, we addressed the hypothesis that the more interesting compounds **6** and **30** are effective also in intact cells. These compounds were selected for studying their antitumor properties in a model of LAMA-84 R cell line over-expressing HO-1 and resistant to imatinib mesylate (IM), a tyrosine-kinase inhibitor used in the treatment of multiple types of cancer, most notably of which includes Philadelphia Chromosome positive (Ph^+) CML.[51]

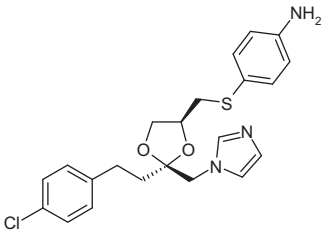
2. Chemistry

Compounds **17–31** were obtained as showed in Scheme 1. In brief, imidazole **7** or 1,2,4-triazole **8** were reacted with appropriate aryloxyalkybromides **9–16** in acetonitrile under microwave irradiation, in the presence of triethylamine (TEA) and tetrabutylammoniumbromide (TBAB) as phase transfer catalyst, at 100 °C, for 30–45 min, to give desired final products **17–31**. Purifications were performed by flash chromatography using ethyl acetate or mixtures of ethyl acetate and methanol. The structures of all new synthesized compounds were confirmed by analytical, IR and ^{1}H NMR spectral data. ^{1}H NMR spectra of triazole derivatives showed two singlets each corresponding in intensity to one magnetically non-equivalent triazole proton, permitting univocally to assign the structure of regioisomers 1,2,4.

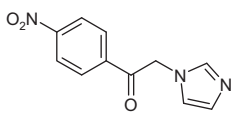
3. Results and discussion

In this work compounds **17–31** were tested to evaluate their ability to inhibit HO-1 obtained from the microsomal fractions of rat spleen. SnPP was used as reference compound. Inhibition of enzyme activity is expressed as IC_{50} (μM) and results are summarized in Table 1. Compounds which showed IC_{50} values <100 μM were also tested on HO-2 obtained from microsomal fractions of rat brain.

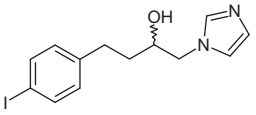
Structural characterization by X-ray crystallography of azole-based inhibitors in complex with HO-1, such as compounds **1** and **4** (Fig. 1), showed that the main features for binding to HO-1 included coordination of a nitrogen present in the azolyl moiety of the inhibitors with the heme iron, and stabilization of the binding through an interaction between hydrophobic groups of the inhibitors and a distal hydrophobic pocket in the heme-binding pocket.[41,42] On the basis of these considerations and starting from the structure of **6**,[48,52] in this work we designed and synthesized new aryloxyalkyl derivatives of imidazole and 1,2,4-triazole, **17–31**, which possess the key chemical features needed for enzyme interaction: (i) an azolyl moiety, represented by imidazole or 1,2,4-triazole, (ii) a hydrophobic group, represented by phenyl or



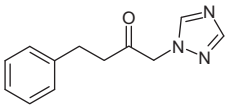
azalanstat (1)
HO-1 IC_{50} = 5.5 μM, HO-2 IC_{50} = 24.5 μM



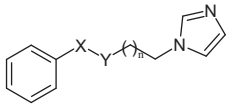
2
HO-1 IC_{50} = 2.5 μM, HO-2 IC_{50} = >100 μM



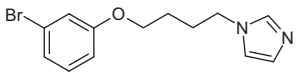
3
HO-1 IC_{50} = 0.06 μM, HO-2 IC_{50} = 1.8 μM



4
HO-1 IC_{50} = 2.6 μM, HO-2 IC_{50} = 34 μM

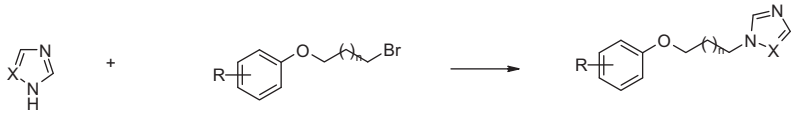


5
X = CH_2, O, S
Y = CH_2, O, S, NH
n = 1, 2



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

Figure 1. Chemical structures and HO-inhibition values of compounds **1–6.**



7,8

7, X = CH
8, X = N

9-16

9, R = H, n = 1
10, R = H, n = 2
11, R = 4-NO_2, n = 1
12, R = 4-NO_2, n = 2
13, R = 3-Br, n = 1
14, R = 3-Br, n = 2
15, R = 4-I, n = 1
16, R = 4-I, n = 2

17-31

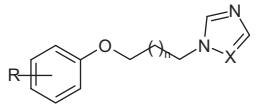
17, R = H, n = 1, X = CH
18, R = H, n = 1, X = N
19, R = H, n = 2, X = CH
20, R = H, n = 2, X = N
21, R = 4-NO_2, n = 1, X = CH
22, R = 4-NO_2, n = 1, X = N
23, R = 4-NO_2, n = 2, X = CH
24, R = 4-NO_2, n = 2, X = N
25, R = 3-Br, n = 1, X = CH
26, R = 3-Br, n = 1, X = N
27, R = 3-Br, n = 2, X = N
28, R = 4-I, n = 1, X = CH
29, R = 4-I, n = 1, X = N
30, R = 4-I, n = 2, X = CH
31, R = 4-I, n = 2, X = N

Scheme 1. Reagents and conditions: TEA, TBAB, acetonitrile, 100 °C, 30–45 min.

substituted (4-NO_2, 3-Br, 4-I) phenyl, chosen having in mind aryl moieties present in imidazole derivatives endowed with good or very good HO inhibitory properties (Fig. 1),[45,47] (iii) a linker, represented by an oxypropyl or oxybutyl chain.

As a general consideration, it is possible to affirm that many of the tested compounds inhibit HO-1 in the micromolar range even if with less potency of related **6**. In particular, compound **30** possesses noteworthy HO-1 inhibition (IC_{50} = 1 μM) and some selectivity towards HO-2 (IC_{50} = 10 μM); this potency, along with that of **6** (HO-1 IC_{50} = 2.1 μM, HO-2 IC_{50} = 2.2 μM) is comparable to that of the first discovered HO inhibitor azalanstat (**1**, Fig. 1, HO-1 IC_{50} = 5.5 μM, HO-2 IC_{50} = 24.5 μM). From the obtained results, SARs can be discussed: the effect of azolyl moiety is very clear since imidazole-containing compounds resulted generally more potent than the corresponding triazole ones (**17** vs **18**, **19** vs **20**, **23** vs **24**, **25** vs **26**, **6** vs **27** and **30** vs **31**). Considering the influence of the length of the linker chain, compounds containing an oxybutyl linker give the best results with respect to those containing an oxypropyl chain (**19** vs **17**, **23** vs **21**, **6** vs **25**, **30** vs **28**, and **31** vs **29**). With regard to the influence of the substituent present in the phenyl group, results show that 4-NO_2 is deleterious, 3-Br gives good results for inhibition of HO-1 but not for selectivity, whereas 4-I gives the best contribution both in terms of potency on HO-1 and selectivity over HO-2. In order to test the influence on activity

Table 1
Inhibitory potency of compounds **17**–**31** against HO-1 and HO-2



Compd	R	*n*	X	IC_{50} HO-1[a]	IC_{50} HO-2[a]
17	H	1	CH	31 ± 2	>100
18	H	1	N	>100	NT
19	H	2	CH	21 ± 1	30 ± 1
20	H	2	N	92 ± 5	27 ± 2
21	4-NO_2	1	CH	>100	NT
22	4-NO_2	1	N	>100	NT
23	4-NO_2	2	CH	56 ± 2	21 ± 1
24	4-NO_2	2	N	>100	NT
25	3-Br	1	CH	69 ± 3	>100
26	3-Br	1	N	>100	NT
27	3-Br	2	N	>100	NT
28	4-I	1	CH	>100	NT
29	4-I	1	N	75 ± 5	90 ± 4
30	4-I	2	CH	1 ± 0.01	10 ± 0.5
31	4-I	2	N	38 ± 1	70 ± 3
32	2-Br	2	CH	53 ± 3	24 ± 3
33	4-Br	2	CH	31 ± 1	75 ± 4
6	3-Br	2	CH	2.1 ± 0.3	2.2 ± 0.2
SnPP				0.58 ± 0.03	0.36 ± 0.01

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

of the position of substituent in the phenyl ring, in this work we also evaluated previously synthesized derivatives **32** and **33**,[52] carrying a 2- and 4-bromophenyl as aryl moieties, respectively. Both compounds result less potent than corresponding 3-bromo analog **6**, towards both HO-1 and HO-2.

Compounds **6** and **30**, showing the highest potency against HO-1, **30** being also 10-fold selective for HO-1 versus HO-2, were selected to further biological studies, in order to explore the potential role of HO-1 inhibitors in antitumor therapy. Moreover we studied the mechanisms involved in their antitumor properties.

It has been reported that different human cancers express high levels of HO-1, which may provide them a growth advantage.[9–15,53] Therefore, inhibition of HO-1 might lead to reduced tumor growth in vitro and in vivo.[21–23,35,54,55] Recent studies demonstrated that induction of HO-1 made leukemia and colon cancer cells resistant to merocyanine and pyrrolidine dithiocarbamate, two experimentally used chemotherapeutic drugs.[56,57] Berberat et al.[15] reported that high HO-1 levels in pancreatic cancer cells may, at least partly, be responsible for their resistance to anticancer therapy.

CML is a stem cell disease in which BCR/ABL [51] promotes the survival of leukemic cells. Identification of IM, a tyrosine-kinase inhibitor used in the treatment of multiple cancers, and the subsequent findings that this compound displays growth inhibitory and pro-apoptotic effects in Bcr–Abl^+ cells, has tremendously modified CML treatment, but the initial striking efficacy of this drug has been overshadowed by the development of clinical resistance.[58–60] Although the majority of Ph^+ CML patients benefit from IM treatment, a substantial number of patients are either initially refractory to treatment (primary resistance) or develop resistance during the course of treatment (secondary or acquired resistance).[61]

Our previous study[62] showed that HO-1 might be involved in the resistance mechanisms of Bcr–Abl^+ cells, contributing to their failure in undergoing apoptosis in the presence of IM. In fact, HO-1 expression resulted higher in IM-resistant K562 and LAMA cell lines, than in IM-sensitive ones. Moreover, in these resistant cells, the combining of siRNA to IM treatment overcome IM resistance.

In the present paper we evaluated the effects of compounds **6** and **30** in a model of LAMA-84 R cell line overexpressing HO-1 and resistant to IM.

Table 2
HO activity in LAMA-84 R cell lysate

	Pmol bilirubin/1 h/mg prot.[a]	% Inhibition
Control	103.8 ± 3.5	
10 μM **6**	49.5 ± 1.2*	52.3
10 μM **30**	47.1 ± 1.8*	54.62

[a] Values are shown from triplicate experiments ± SD.
* Significant versus untreated control cells: p <0.001

Results reported in Table 2 evidence that compounds **6** and **30** at 10 μM concentration are able to inhibit HO activity in LAMA-84 R cell line respect to untreated cells. Therefore they are effective not only in broken-cell preparation, such as the above-described microsomal fractions, but also in intact cells, suggesting that they are able to cross the cellular membrane and that could be active in vivo.

Moreover we evaluated if the HO-1 inhibition induced by compounds **6** and **30** was able to overcome IM resistance. This hypothesis is confirmed by data reported in Figure 2. Results evidence that the single treatment with IM or compound **6** or compound **30** does not modify percentage of cell survival whereas treatment of LAMA-84 R cell line with compounds **6** or **30** plus IM is able to reduce percentage of cell survival.

According to these results, Fluorescence Activated Cell Sorting (FACS) analysis reveals that only compounds **6** or **30** (10 μM) plus IM (1 μM) treatment, but not the single treatment with IM or compound **6** or **30**, induces decrease of S and G_2/M phases with increased percentage of apoptotic cells (Fig. 3).

In our previous study[62] we reported that HO-1 mRNA levels were significantly higher in LAMA-84 resistant cells when compared to non-resistant clones. In the present paper we measured HO-1 and HO-2 mRNA levels in LAMA-84 resistant cells treated and untreated with compounds **6** or **30**. It is interesting to evidence that compounds **6** or **30** are able to decrease HO-1 mRNA levels whereas they don't modify HO-2 mRNA levels (Fig. 4). Thus, compounds **6** and **30**, contrarily to Mps and other HO-1 inhibitors, do not behave as very potent HO-1 inducers but they are able both to down-regulate HO-1 expression and to inhibit HO-1/HO-2 activity.

Datta et al. reported that down-regulation of HO-1 expression lead to cellular apoptosis.[63] According to these data, compounds **6** or **30** plus IM treatment lead to cellular apoptosis.

Moreover we reported that HO-1 siRNA abolished IM resistance in LAMA-84 R cell population, restoring apoptotic effects of IM.[62]

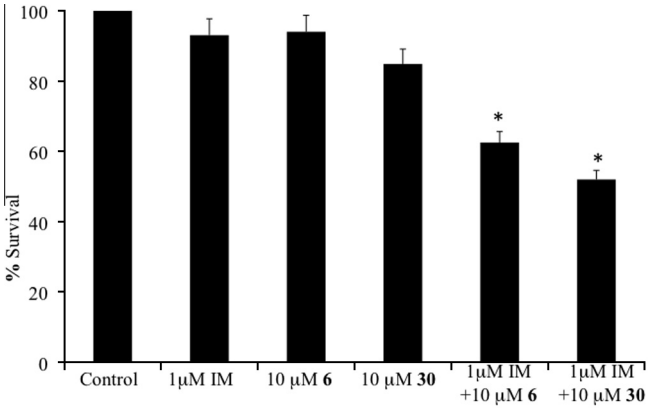


Figure 2. Effects of 1 μM IM alone, or 10 μM **6** alone, or 10 μM **30** alone, or a combination of drugs. Survival analyzed by ATP lite assay. Values are shown from triplicate experiments ± SD. *Significant versus untreated control cells and versus 1 μM IM alone, or 10 μM **6** alone, or 10 μM **30** alone treated cells: p <0.05.

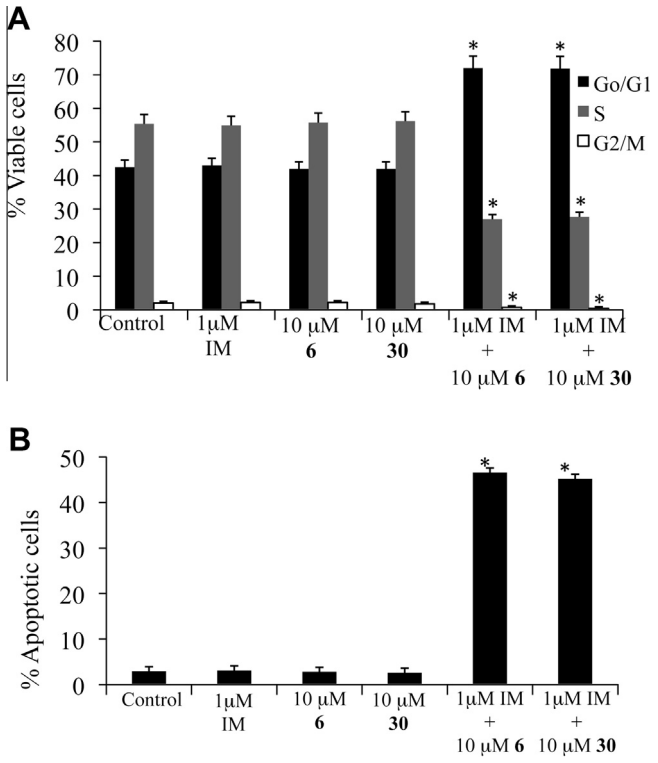


Figure 3. Cell cycle distribution of survival cells (Panel A) and apoptotic cells (Panel B) analyzed by FACS. Values are shown from triplicate experiments ± SD. *Significant versus untreated control cells and versus 1 μM IM alone, or 10 μM **6** alone, or 10 μM **30** alone treated cells: p <0.001.

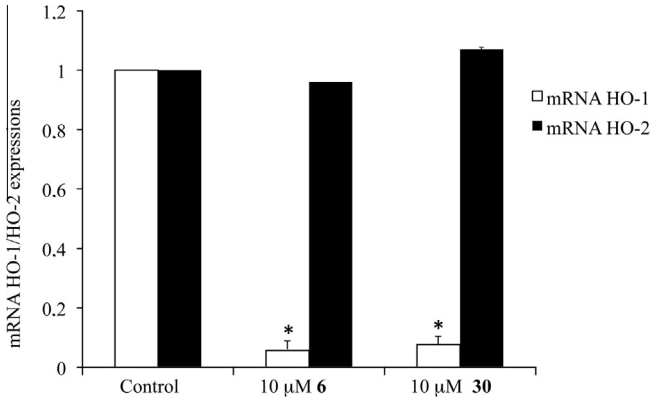


Figure 4. Effect of 10 μM **6** and 10 μM **30** on mRNA expression analyzed by Real Time PCR. Values are shown from triplicate experiments ± SD. *Significant versus untreated control cells: p <0.05.

Since apoptotic effect of IM may be mediated by ROS formation,[62] here we measured the levels of intracellular oxidants in LAMA-84 R cells untreated and treated with IM alone, or IM combined with **6** or **30**. Figure 5 shows that co-administration of IM and both compounds increases ROS levels. This effect may be related to increased percentage of apoptotic cells mediated by compounds **6** or **30** plus IM treatment. Therefore, the inhibition of HO-1 may allow the IM mediated generation of ROS and the elevated ROS levels may facilitate tumor killing.

Overall results reported in the present paper demonstrate that compounds **6** or **30** plus IM treatment can down-regulate HO-1 expression and, in this condition, IM can cause an increase of ROS levels with consequent tumor cell apoptosis.

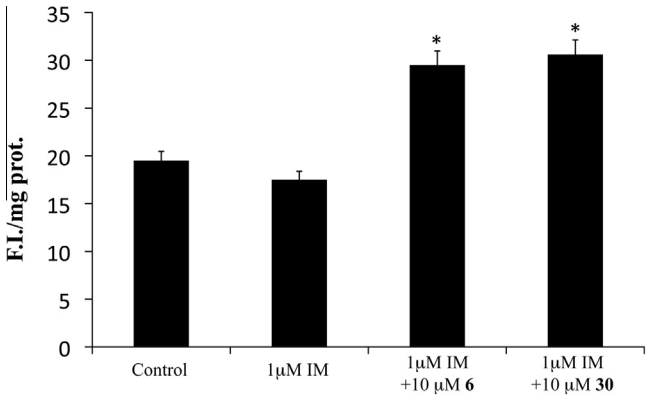


Figure 5. Intracellular oxidants in LAMA-84 R cells untreated and treated with 1 μM IM alone, or 1 μM IM + 10 μM **6**, or 1 μM IM + 10 μM **30**. Values are shown from triplicate experiments ± SD. *Significant versus untreated control cells and versus 1 μM IM alone treated cells: p <0.05.

4. Conclusions

In the present study, a novel series of aryloxyalkyl derivatives of imidazole and 1,2,4-triazole, **17**–**31**, was investigated as HO-inhibitors. Best results were obtained for imidazole derivatives carrying an oxybutyl chain as linker. Particularly, 1-[4-(3-bromophenoxy)butyl]-1*H*-imidazole **6** and 1-[4-(4-iodophenoxy)butyl]-1*H*-imidazole **30** showed the highest inhibitory potency against HO-1; **30** resulted moderately selective for HO-1 versus HO-2.

This effect was confirmed also in LAMA-84 R cell line, demonstrating the capacity of these compounds to be active also in intact cells. Moreover compounds **6** and **30** besides to inhibit HO-1/HO-2 activity are able to down-regulate HO-1 expression and in this condition, in LAMA-84 R cell line, IM can cause an increase of ROS levels with consequent cell apoptosis. Therefore, simultaneous administration of **6** or **30** with IM sensitized LAMA-84 R cell line to IM.

In conclusion, we suggest that specific HO-1 activity decrease mediated by HO-1 inhibitors, such as **6** and **30**, in combination with conventional radiotherapy and chemotherapy may open up new perspectives in the treatment of CML.

5. Experimental procedures

5.1. Chemistry

Melting points were determined in an Electrothermal IA9200 apparatus with a digital thermometer in glass capillary tubes. Elemental analyses for C, H, N were within ± 0.4% of theoretical values and were performed on a Carlo Erba Elemental Analyzer 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 F_{254}) 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). All chemicals and solvents were reagent grade and were purchased from commercial sources. Aryloxyalkylbromides **11**, **13**–**16** were synthesized following described procedures[64] with slight modifications, in brief: corresponding phenols (5 mmol) were reacted

with 1,3-dibromopropane or 1,4-dibromobutane (10 mmol) and potassium carbonate (10 mmol) in acetone (3 mL), under microwaves irradiation in a sealed vial at 90 °C, 200 W, 150 Psi, for 30 min. The solvent was removed in vacuum to give a residue which was suspended in water and extracted with dichloromethane (3 × 50 mL); the combined extracts were washed with water, dried, and evaporated to obtain a residue which was used without any further purification. Analytical and spectral data of previous synthesized 1-[4-(3-bromophenoxy)butyl]-1*H*-imidazole **(6)** [52] are: colorless oil; yield 32%; IR (Neat) cm^{-1}: 3112, 2913, 1589, 1488, 1228, 780; ^1H NMR ($CDCl_3$) δ 7.49 (br s, 1H, imidazole), 7.18–7.02 (m, 3H aromatic + 1H imidazole), 6.93 (br s, 1H, imidazole), 6.83–6.77 (m, 1H, aromatic), 4.06–3.91 (m, 4H, OCH_2 + NCH_2), 2.06–1.94 (m, 2H, OCH_2CH_2), 1.83–1.73 (m, 2H, NCH_2CH_2). Anal. $C_{13}H_{15}BrN_2O$ (C,H,N). Analytical and spectral data of all unknown compounds are following reported.

5.2. General procedure for the synthesis of aryloxyalkyl derivatives of imidazole or 1,2,4-triazole (17–31)

A solution of the appropriate aryloxyalkylbromides **9**–**16** (5 mmol) commercially available or prepared as above-described, imidazole **7** or 1,2,4-triazole **8** (7.5 mmol), TEA (5 mmol) and catalytic amount of TBAB (0.1 g) in acetonitrile (3 mL) was heated under microwaves irradiation in a sealed vial at 100 °C, 200 W, 150 Psi, for 30–45 min. The solvent was removed in vacuo to give a residue which was suspended in water, alkalinized with NaOH 0.1 N and extracted with dichloromethane (3 × 50 mL); the combinated extracts were washed with water, dried, and evaporated to obtain a residue which was purified by flash column chromatography on silica gel using ethyl acetate or ethyl acetate/methanol 9:1 as eluent. By use of this procedure, the subsequent compounds were obtained:

5.2.1. 1-(3-Phenoxypropyl)-1*H*-imidazole (17)

Analytical and spectral data were in agreement with Ref. 65.

5.2.2. 1-(3-Phenoxypropyl)-1*H*-1,2,4-triazole (18)

The title compound was isolated as colourless oil; yield 28%; IR (Neat) cm^{-1}: 3117, 2948, 1600, 1497, 1244, 757; ^1H NMR (DMSO-d_6) δ 8.53 (s, 1H, triazole), 7.98 (s, 1H, triazole), 7.32–7.24 (m, 2H, aromatic), 6.96–6.89 (m, 3H, aromatic), 4.36 (t, J = 6.8 Hz, 2H, OCH_2), 3.94 (t, J = 6.2 Hz, 2H, NCH_2), 2.26–2.19 (m, 2H, $CH_2CH_2CH_2$). Anal. $C_{11}H_{13}N_3O$ (C,H,N).

5.2.3. 1-(4-Phenoxybutyl)-1*H*-imidazole (19)

Analytical and spectral data were in agreement with Ref. 65.

5.2.4. 1-(4-Phenoxybutyl)-1*H*-1,2,4-triazole (20)

The title compound was isolated as white solid; yield 25%; mp 48–49 °C; IR (KBr) cm^{-1}: 3101, 2947, 1602, 1499, 1255, 755; ^1H NMR (DMSO-d_6) δ 8.55 (s, 1H, triazole), 7.98 (s, 1H, triazole), 7.32–7.24 (m, 2H, aromatic), 6.95–6.88 (m, 3H, aromatic), 4.25 (t, J = 6.8 Hz, 2H, OCH_2), 3.96 (t, J = 6.2 Hz, 2H, NCH_2), 1.98–1.90 (m, 2H, OCH_2CH_2), 1.70–1.62 (m, 2H, NCH_2CH_2). Anal. $C_{12}H_{15}N_3O$ (C,H,N).

5.2.5. 1-[3-(4-Nitrophenoxy)propyl]-1*H*-Imidazole (21)

The title compound was isolated as light yellow solid; yield 34%; mp 76–78 °C. IR (KBr) cm^{-1}: 3103, 2950, 1593, 1504, 1339, 1258; ^1H NMR (DMSO-d_6) δ 8.22–8.17 (m, 2H, aromatic), 7.63 (s, 1H, imidazole), 7.20–7.09 (m, 2H aromatic + 1H imidazole), 6.89 (br s, 1H, imidazole), 4.15 (t, J = 7.0 Hz, 2H, OCH_2), 4.05 (t, J = 6.2 Hz, 2H, NCH_2), 2.27–2.14 (m, 2H, $CH_2CH_2CH_2$). Anal. $C_{12}H_{13}N_3O_3$ (C,H,N).

5.2.6. 1-[3-(4-Nitrophenoxy)propyl]-1*H*-1,2,4-triazole (22)

The title compound was isolated as white solid; yield 25%; mp 115–117 °C; IR (KBr) cm^{-1}: 3136, 2968, 1592, 1502, 1330, 1262; ^1H NMR (DMSO-d_6) δ 8.54 (s, 1H, triazole), 8.25–8.17 (m, 2H, aromatic), 7.98 (s, 1H, triazole), 7.16–7.08 (m, 2H, aromatic), 4.37 (t, J = 6.8 Hz, 2H, OCH_2), 4.12 (t, J = 6.0 Hz, 2H, NCH_2), 2.34–2.21 (m, 2H, $CH_2CH_2CH_2$). Anal. $C_{11}H_{12}N_4O_3$ (C,H,N).

5.2.7. 1-[4-(4-Nitrophenoxy)butyl]-1*H*-Imidazole (23)

The title compound was isolated as yellow solid; yield 27%; mp 67–69 °C; IR (KBr) cm^{-1}: 3087, 2938, 1591, 1500, 1331, 1269; ^1H NMR (DMSO-d_6) δ 8.24–8.16 (m, 2H, aromatic), 7.65 (s, 1H, imidazole), 7.20–7.09 (m, 2H aromatic + 1H imidazole), 6.89 (br s, 1H, imidazole), 4.12 (t, J = 6.4 Hz, 2H, OCH_2), 4.04 (t, J = 6.8 Hz, 2H, NCH_2), 1.94–1.61 (m, 4H, $CH_2CH_2CH_2CH_2$). Anal. $C_{13}H_{15}N_3O_3$ (C,H,N).

5.2.8. 1-[4-(4-Nitrophenoxy)butyl]-1*H*-1,2,4-triazole (24)

The title compound was isolated as white solid; yield 28%; mp 90–91 °C; IR (KBr) cm^{-1}: 3114, 2942, 1590, 1500, 1335, 1254; ^1H NMR (DMSO-d_6) δ 8.55 (s, 1H, triazole), 8.23–8.16 (m, 2H, aromatic), 7.98 (s, 1H, triazole), 7.18–7.09 (m, 2H, aromatic), 4.26 (t, J = 6.8 Hz, 2H, OCH_2), 4.14 (t, J = 6.2 Hz, 2H, NCH_2), 1.99–1.88 (m, 2H, OCH_2CH_2), 1.77–1.67 (m, 2H, NCH_2CH_2). Anal. $C_{12}H_{14}N_4O_3$ (C,H,N).

5.2.9. 1-[3-(3-Bromophenoxy)propyl]-1*H*-imidazole (25)

The title compound was isolated as colorless oil; yield 21%; IR (Neat) cm^{-1}: 3110, 2938, 1588, 1468, 1284, 1228; ^1H NMR (DMSO-d_6) δ 7.62 (s, 1H, imidazole), 7.28–7.10 (m, 3H aromatic + 1H imidazole), 6.97–6.88 (m, 1H aromatic + 1H imidazole), 4.12 (t, J = 7.0 Hz, 2H, OCH_2), 3.91 (t, J = 6.0 Hz, 2H, NCH_2), 2.21–2.08 (m, 2H, $CH_2CH_2CH_2$). Anal. $C_{12}H_{13}BrN_2O$ (C,H,N).

5.2.10. 1-[3-(3-Bromophenoxy)propyl]-1*H*-1,2,4-triazole (26)

The title compound was isolated as pale yellow oil; yield 20%; IR (Neat) cm^{-1}: 3117, 2947, 1589, 1468, 1274, 1228; ^1H NMR (DMSO-d_6) δ 8.53 (s, 1H, triazole), 7.97 (s, 1H, triazole), 7.28–6.90 (m, 4H, aromatic), 4.34 (t, J = 6.8 Hz, 2H, OCH_2), 3.97 (t, J = 6.0 Hz, 2H, NCH_2), 2.28–2.15 (m, 2H, $CH_2CH_2CH_2$). Anal. $C_{11}H_{12}BrN_3O$ (C,H,N).

5.2.11. 1-[4-(3-Bromophenoxy)butyl]-1*H*-1,2,4-triazole (27)

The title compound was isolated as pale yellow oil; yield 23%; IR (Neat) cm^{-1}: 3117, 2946, 1589, 1469, 1228, 680; ^1H NMR (DMSO-d_6) δ 8.52 (s, 1H, triazole), 7.97 (s, 1H, triazole), 7.27–6.90 (m, 4H, aromatic), 4.24 (t, J = 6.8 Hz, 2H, OCH_2), 3.99 (t, J = 6.4 Hz, 2H, NCH_2), 1.99–1.85 (m, 2H, OCH_2CH_2), 1.71–1.60 (m, 2H, NCH_2CH_2). Anal. $C_{12}H_{14}BrN_3O$ (C,H,N).

5.2.12. 1-[3-(4-Iodophenoxy)propyl]-1*H*-imidazole (28)

The title compound was isolated as white solid; yield 23%; mp 87–88 °C; IR (KBr) cm^{-1}: 3092, 2944, 1584, 1487, 1229, 824; ^1H NMR (DMSO-d_6) δ 7.62–7.54 (m, 2H, aromatic + 1H imidazole), 7.18 (br s, 1H, imidazole), 6.88 (br s, 1H, imidazole), 6.81–6.75 (m, 2H aromatic), 4.11 (t, J = 7.0 Hz, 2H, OCH_2), 3.87 (t, J = 6.2 Hz, 2H, NCH_2), 2.21–2.11 (m, 2H, $CH_2CH_2CH_2$). Anal. $C_{12}H_{13}IN_2O$ (C,H,N).

5.2.13. 1-[3-(4-Iodophenoxy)propyl]-1*H*-1,2,4-triazole (29)

The title compound was isolated as white solid; yield 21%; mp 77–79 °C; IR (KBr) cm^{-1}: 3103, 2944, 1585, 1508, 1487, 1236; ^1H NMR (DMSO-d_6) δ 8.51 (s, 1H, triazole), 7.97 (s, 1H, triazole), 7.62–7.54 (m, 2H, aromatic), 6.80–6.72 (m, 2H, aromatic), 4.34 (t, J = 6.8 Hz, 2H, OCH_2), 3.93 (t, J = 6.0 Hz, 2H, NCH_2), 2.28–2.15 (m, 2H, $CH_2CH_2CH_2$). Anal. $C_{12}H_{12}IN_3O$ (C,H,N).

5.2.14. 1-[4-(4-Iodophenoxy)butyl]-1*H*-imidazole (30)

The title compound was isolated as white solid; yield 16%; mp 90–92 °C; IR (KBr) cm^{-1}: 3112, 2942, 1586, 1488, 1468, 1254; ^1H NMR (DMSO-d_6) δ 7.62–7.53 (m, 2H, aromatic + 1H imidazole), 7.17 (br s, 1H, imidazole), 6.88 (br s, 1H, imidazole), 6.80–6.73 (m, 2H, aromatic), 4.04–3.90 (m, 4H, OCH_2 + NCH_2), 1.90–1.76 (m, 2H, OCH_2CH_2), 1.68–1.58 (m, 2H, NCH_2CH_2). Anal. $C_{13}H_{15}IN_2O_3$ (C,H,N).

5.2.15. 1-[4-(4-Iodophenoxy)butyl]-1*H*-1,2,4-triazole (31)

The title compound was isolated as pale yellow solid; yield 19%; mp 57–58 °C; IR (KBr) cm^{-1}: 3104, 2913, 1585, 1511, 1487, 1247: ^1H NMR (DMSO-d_6) δ 8.52 (s, 1H, triazole), 7.96 (s, 1H, triazole), 7.61–7.53 (m, 2H, aromatic), 6.81–6.73 (m, 2H, aromatic), 4.23 (t, J = 6.8 Hz, 2H, OCH_2), 3.95 (t, J = 6.4 Hz, 2H, NCH_2), 1.98–1.84 (m, 2H, OCH_2CH_2), 1.67–1.60 (m, 2H, NCH_2CH_2). Anal. $C_{12}H_{14}$ IN_3O (C,H,N).

6. Biology

6.1. 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.[47,66–68] 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.[1] 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.[48]

6.2. Preparation of biliverdin reductase

Liver cytosol has been used as a source of biliverdin reductase (BVR). 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.

6.3. 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.[1] 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, 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 1 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.

6.4. Cell cultures and treatments

Ph^+ cell lines LAMA-84 R, were cultured in RPMI-1640 (Sigma, Milan, Italy) with 10% Fetal Bovine Serum (FBS) and 1% penicillin–streptomycin at a final concentration of 15 × 10^4 cells/mL and were incubated at 37 °C in 5% CO_2. Cells were incubated for 24 h either with IM 1 μM alone, or 10 μM compound **6** alone, or 10 μM compound **30** alone, or a combination of drugs.

6.5. ATP-lite1step assay for cell survival

After drug treatment, the viability of cells was evaluated by the ATP-lite1step assay (PerkinElmer, Monza, Italy), as described by the manufacturer. Briefly, cells were plated onto 96-well microplates in 100 μL growth medium and 100 μL of the reconstituted reagent was added to each well. Cells were incubated at 37 °C for 20 min at 700 rpm using an orbital microplate shaker. Luminescence was measured using a Victor3 (PerkinElmer). Drug-resistance was calculated as the percentage of viable cells in the treated suspension culture in comparison to the untreated one.

6.6. Cell cycle analysis

Cells were washed and resuspended in cold 80% ethanol to a final concentration of 0.5 × 10^6 cells/mL for 1 h at 4 °C. The ethanol-fixed cells were centrifuged to remove ethanol and the pellet was resuspended in propidium iodide staining reagent (0.1% triton X-100, 0.1 mM EDTA, 0.05 mg/mL RNase A and 50 μg/mL propidium iodide). Cells were stored in the dark at room temperature for about 3 h. Cells were then analyzed with a FACS flow cytometer (FC500 Beckman coulter; Beckman Coulter S.p.a., Milan, Italy) and processed by the ModFit program. The treated cells were evaluated by FACS analysis for identifying the cells at different stages of the cell cycle.

6.7. Real-time PCR quantification

Expression of HO-1/HO-2 and GAPDH were evaluated by real-time PCR. Cultured cell layers in different conditions were rinsed with cold PBS and immediately lysed using Trizol Reagent (Qiagen). Total RNA was isolated, treated with RNase-free DNase I, and quantified by UV spectrophotometry. For RT-PCR analysis of mRNA expression, 1.0 μg of total RNA (in 20 μL reaction volume) was reverse-transcribed using reverse transcriptase (Roche Diagnostic) and oligo-dT primers in a standard reaction. The resultant cDNA was then used as the template for PCR amplification. The quantitative real-time polymerase chain reaction (qRT-PCR) was

performed with the TaqMan gene expression assay (HMOX1 Hs00157965_m1; HMOX2 Hs01558390_m1; GAPDH Hs99999905_m1 Life Technologies) on an ABI Prism 7900 sequence analyzer according to the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA, USA). Each reaction was run in triplicate. The comparative threshold cycle (CT) method was used to calculate the amplification fold as specified by the manufacturer. A value of 10 ng of reverse transcribed RNA samples was amplified by using the TaqMan Universal PCR Master Mix.

6.8. Measurement of HO enzymatic activity in LAMA-84 R cell line

Total HO activity in the cell lysate was determined by measuring the bilirubin formation using the difference in absorbance at 464–530 nm as described by Ryter et al.[1]

Reaction mixtures (500 μL) consisted of 20 mM Tris–HCl, pH 7.4, (1 mg/mL) cell lysate, 0.5–2 mg/mL biliverdin reductase, 1 mM NADPH, 2 mM glucose 6-phosphate (G6P), 1 U G6P dehydrogenase, 25 μM hemin, 10 μL of DMSO (or the same volume of DMSO solution of compounds **6** or **30** to a final concentration of 10 μM). Incubations were carried out for 60 min at 37 °C in a circulating water bath in the dark. Reactions were stopped by adding 1 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.

6.9. ROS measurement

Determination of ROS was performed by using a fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA), as previously described.[69] The fluorescence [corresponding to the oxidised radical species 2′,7′-dichlorofluorescein (DCF)] was monitored spectrofluorometrically (excitation, $\lambda = 488$ nm; emission, $\lambda = 525$ nm). The total protein content was evaluated for each sample and the results are reported as fluorescence intensity/mg protein.

6.10. Statistical analyses

The data are presented as means ± SD for 4 experiments in triplicate. One-way variance analysis and Student's *t*-test were used where appropriate; $p < 0.05$ was regarded as significant.

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