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Research paper

Protective effect of piceatannol and bioactive stilbene derivatives against hypoxia-induced toxicity in H9c2 cardiomyocytes and structural elucidation as 5-LOX inhibitors

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ABSTRACT

Stilbenes with well-known antioxidant and antiradical properties are beneficial in different pathologies including cardiovascular diseases. The present research was performed to investigate the potential protective effect of **resveratrol (1)** and **piceatannol (2)**, against hypoxia-induced oxidative stress in the H9c2 cardiomyoblast cell line, and the underlying mechanisms. Compounds **1** and **2** significantly inhibited the release of peroxynitrite and thiobarbituric acid levels at na no- or submicromolar concentrations, and this effect was more evident in piceatannol-treated cells, that significantly increased MnSOD protein level in a concentration dependent manner. Furthermore, since piceatannol, which is far less abundant in natural sources, displayed a higher bioactivity than the parent compound, we hereby report on a very fast synthesis and detailed structure-based design of a focused stilbene library. Finally, taking into account that hypoxia-induced ROS accumulation also increases expression and activity of 5-lipoxygenase (5-LOX) with production of leukotrienes, we have disclosed structural key factors crucial for 5-LOX activity. Among the synthesized analogues (**3**–**7**), compound **7** was the most effective in improving cardiomyocytes viability and in 5-LOX inhibition. In conclusion, modeling and experimental studies provided the basis for further optimization of stilbene analogues as multi-target inhibitors of the inflammatory and oxidative pathway.

1. Introduction

Hypoxia-induced oxidative stress and cardiomyocyte apoptosis are considered as essential processes in the progression of heart failure. Hypoxia, caused by an imbalance in the demand and supply of oxygen, often occurs in physiologic conditions such as high altitude and in pathological conditions including ischemia and cardiovascular disorders in which the heart is very sensitive due to

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its high oxygen consumption [1,2]. Sufficient supply of oxygen is essential for the proper functioning of cardiomyocytes [3]. Many studies indicated that hypoxia has adverse effects on myocardial cells [4–6], but the mechanism involved in the occurrence of hypoxia injury is far from being fully understood. Cell hypoxia is associated with the generation of reactive oxygen species (ROS). Increased amounts of ROS, generated via NADPH oxidase, mitochondrial electron transport, xanthine oxidase, lipoxygenases, and nitric oxide synthase (NOS) can lead to apoptosis, resulting in lethal cell injuries [7].

Thus, by focusing on the scavenging of ROS as a promising therapeutic target for hypoxia injury, antioxidant compounds have been of great interest as potential therapeutic compounds for hypoxia-induced oxidative stress. In this regard, nature has always been a fascinating source of interest to continually discover new substances [8]. Many antioxidant compounds were isolated from plant extracts, including polyphenolic derivatives, which were studied to clarify their mechanism of action [9]. On that basis, research efforts on the human health effects of natural polyphenols has grown in the last 50 years [10]. Studies have brought to light the strong contribution of polyphenols in prevention and treatment of cardiovascular pathologies, cancer, osteoporosis, diabetes mellitus and neurodegenerative disorders such as Parkinson's and Alzheimer's disease [11–15].

Although polyphenols are mainly of natural origin, it is possible to develop synthetic substances mimicking natural counterparts since they can be relatively inexpensive, easily accessible and with highly reproducible biological properties, including antioxidant activity [16]. Note that biological activity of polyphenols is determined by several factors; the most important is related to direct interaction with receptors and enzymes involved in signal transduction that can modify the redox status of cells and promote a series of redox-dependent reactions in addition to the possibility of express the well-known antioxidant and free radicals scavenging activity, which protect cells against oxidative damage [17,18]. The main antioxidants are characterized by the presence of phenolic groups in their chemical structure, particularly, catechol, pyrogallol or hydroquinone groups [19].

Hydroquinones and catechols are very easily converted to quinones by oxidation catalysed by virtually any oxidative enzyme or metal ions, in this context the biological activities of this class of compounds can be correlated with the known properties of the parent compound(s) [20–25].

Recent studies focused their attention on stilbenoids that are poly-hydroxy natural products with resveratrol representing one of the highest exponents of the class [26]. *Trans*-resveratrol (*trans*-3′,4,5′-trihydroxystilbene), is a natural phytoalexin identified for the first time in the wine grape *Vitis vinifera* in 1976, and today has been found in more than 70 plant species. It is often related to so-called “French paradox”, the phenomenon whereby in France, despite the high consumption of saturated fatty acids rich food, drinking of red wine reduces the incidence of mortality for cardiovascular diseases [27–30]. The biological properties of resveratrol are described in detail in several reviews [31–37]. Its beneficial properties continuously inspired researchers to search new and more effective analogues [38–41].

Piceatannol (*trans*-2,3′,4’,5-tetrahydroxystilbene, also known as *Astringine*), a hydroxy analogue of resveratrol, is known as the least abundant in nature. It has been identified only in some species: *Melaleuca leucadendron* (White tea tree), *Cassia garretiana* (Asian legume), *Rheum undulatum* (Corean rhubarb) and in cell culture suspension of *Vitis vinifera*. [42] Having a catechol group, piceatannol might have more biological activities than the analogous structures. It is a potent antioxidant and an anti-leukemic substance, shows anti-allergic effects and induces cellular apoptosis [43].

Studies have also suggested that some of the activities of resveratrol are, in fact, related to its biotransformation into piceatannol by the enzyme CYP1B1 of the cytochrome P450 family [44,45]. Among its many activities, piceatannol, the “less-known congener” of the famous resveratrol, can help preventing the development and progression of cardiovascular diseases (CVDs) through multiple mechanisms such as inhibition of low-density lipoprotein cholesterol (LDL-c) oxidation, inhibition of platelet aggregation, mediation of cardiac cell function, and reduction of myocardial tissue damage during ischemic events by anti-inflammatory activity [46].

During pathologic states such as ischemia or congestive heart failure, eicosanoids contribute to multiple maladaptive changes including inflammation, alterations of cellular growth programs, and activation of multiple transcriptional events leading to the deleterious sequel of these pathologic states. Myocardial phospholipids serve as primary reservoirs of arachidonic acid (AA), which is liberated through the rate-determining hydrolytic action of cardiac phospholipases A_2 ($PLA_2\beta$). AA released by PLA_2-catalysed hydrolysis of phospholipids serves as a precursor for eicosanoids generated by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) pathways [47]. The metabolism of AA in the heart is primarily determined by three cell types (i.e. myocardial, endothelial, and vascular smooth muscle), which rely on complex intercellular communication through paracrine signalling to coordinate blood flow, contractile state, and haemodynamic function [48].

Inflammatory cells such as neutrophils or monocytes can penetrate acutely into the myocardium following ischemic damage and produce a wide variety of biologically active oxidized eicosanoids. For example, the LOX products 5-and 12-hydroxyeicosatetraenoic acid (5-HETE and 12-HETE) levels were found to dramatically increase in cultured canine myocytes following 45 min of hypoxia and 5 h re-oxygenation [49]. Similarly, ischemic rabbit myocardium produced greater amounts of leukotriene B_4, 5-HETE, and 12-HETE than non-ischemic controls, with the latter being the major product [50].

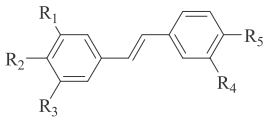
The roles of LOXs and their products in myocardial function remain relatively unexplored, nowadays inhibition of 5-LOX is known for the treatment of asthma [51], but initial investigations have revealed an up-regulation of LOX enzymes and/or LOX products following myocardial ischemia/infarction which may contribute to cardiac hypertrophy, myocyte apoptosis, and fibrosis.

Therefore, the purpose of the current study was to investigate the effects of resveratrol and piceatannol on H9c2 cardiomyocytes subjected to hypoxia-induced oxidative stress. To this end, we have investigated the oxidative balance between ROS release and antioxidant defence, moreover, taking into account that Hypoxia-induced ROS accumulation also increases expression and activity of 5-LOX in pulmonary artery endothelial cells with production of leukotrienes and induction of cell proliferation [49] we have evaluated also their potential as direct inhibitors of 5-LOX.

Our groups have long been involved in a variety of drug discovery projects involving painstaking molecular-level syntheses and modifications as the basic components of research and development, using lead structures that may still have suboptimal structural features requiring modification to fit better to their respective target(s) [52–58].

To this end, considering piceatannol our lead structure and further extend our verification of the inhibitory effect, we synthesize novel stilbene analogues (Table 1) and evaluated their 5-LOX inhibitory activity and protective effects on cardiomyocytes viability *vs* the natural lead.

Table 1
Structures of resveratrol, piceatannol and stilbene structural analogues 1-7.

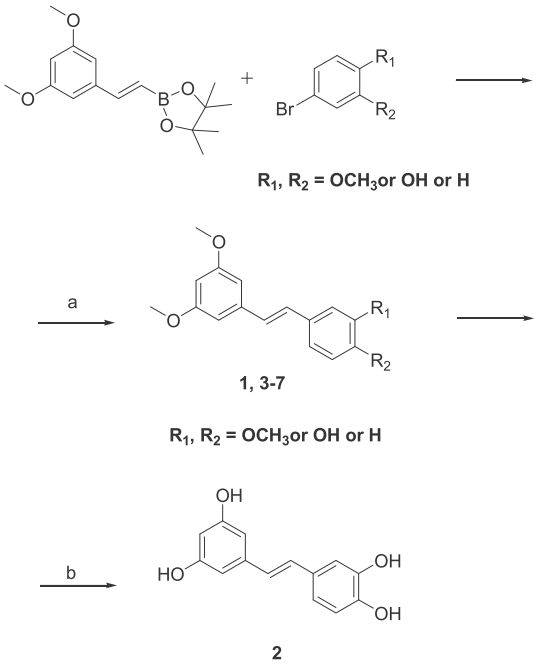


Compd.	Pos.3 (= R_1)	Pos.4 (= R_2)	Pos.5 (= R_3)	Pos.3′(= R_4)	Pos.4′(= R_5)
1 (Resveratrol)	−OH	−H	−OH	−H	−OH
2 (Piceatannol)	−OH	−H	−OH	−OH	−OH
3	$-OCH_3$	−H	$-OCH_3$	−H	−OH
4	$-OCH_3$	−H	$-OCH_3$	−H	$-OCH_3$
5	$-OCH_3$	−H	$-OCH_3$	−OH	$-OCH_3$
6	$-OCH_3$	−H	$-OCH_3$	$-OCH_3$	−OH
7	$-OCH_3$	−H	$-OCH_3$	−OH	−OH

2. Results and discussion

2.1. Chemistry

Methoxylated (**3**–**7**) piceatannol and resveratrol structural analogues were synthesized using standard chemical methodologies, under microwave irradiation by Suzuki-coupling conditions. Essentially, 1 mmol of the corresponding brominated compound were added to a solution of 1.2 mmol of *trans*-2-(3,5-dimethoxyphenyl)-vinylboronic acid pinacol ester (obtained from a commercial supplier and was of analytical grade) in EtOH in presence of catalytic amount of [1,1′ bis(diphenylphosphino) ferrocene] dichloropalladium (II) and K_2CO_3. The reaction mixture was stirred and irradiated by microwave at 110 °C for 30 min to yield the desired methoxystilbenes. Finally, demethylation of the phenolic ethers (6) was successfully established with boron tribromide at room temperature to obtain compound 2 (Scheme 1). Those procedures afforded all desired compounds in a good yield.



Scheme 1. General synthetic route for synthesis of the resveratrol (1) and piceatannol (2) analogues 3–7. Reagents and conditions: (a) dppf (0.005 mmol), K_2CO_3 (2 mmol), EtOH, 110 °C, 30′, MW; (b) BBr_3, CH_2Cl_2, rt.

2.2. Evaluation of anti-oxidant activity against hypoxia-induced oxidative stress in H9c2 cells

Hypoxia-induced oxidative stress and the resulting cell apoptosis are central in the pathogenesis of heart failure [13–15]. H9c2 cells are rat embryonic myoblast cells and retain several characters similar to cardiac cells and have been extensively used as *in vitro* models to study cardiac ischemia/hypoxia [16–18]. In this study, we observed that H9c2 cells were highly sensitive to hypoxia-induced injury in terms of loss of cell viability. In fact, 48 h of hypoxia reduced H9c2 viability by 30% compared to normoxic control cells. Eighteen hours pretreatment and co-treatment with different concentrations of resveratrol (compound **1**), ranging from 80 nM to 7 μM, significantly recovered cell viability up to 95%. Indeed, 20 μM compound 1 was cytotoxic in both treatment conditions (~80% cell death) (Fig. 1A). In the same way, 18 h pretreatment and co-treatment with different concentrations of piceatannol (compound **2**), ranging from 80 nM to 7 μM, significantly recovered cell viability (Fig. 1B). A concentration of 20 μM compound **2** in both treatment conditions drastically reduced cell viability (~80%) (Fig. 1B). On the basis of these results, we chose to perform the next experiments with a pre-treatment protocol to mimic a condition of prevention care in hypoxia-induced disease, such as myocardial infarction.

Hypoxia is best defined as an imbalance between supply and demand for oxygen. In particular, under normal cellular conditions there is a constant balancing act between the production of ROS and their degradation by antioxidant systems including enzymes such as superoxide dismutase, glutathione peroxidase, and catalase. Hearts of mice overexpressing the antioxidant enzyme superoxide dismutase present reduced infarct size associated with increased superoxide radical removal after an ischemia-reperfusion event compared with non-transgenic controls [59].

To evaluate the anti-oxidant activity of resveratrol and piceatannol in our experimental hypoxic system we measured the release of peroxynitrite and the levels of lipid peroxidation in cardiomyocytes exposed to 48 h hypoxia compared to normoxic cells. Forty-eight hours of hypoxia induced significant release of peroxynitrite compared to normoxic control cells. 80 nM and 2 μM pretreatment with resveratrol (**1**) or piceatannol (**2**) significantly reduced NO release. This effect was more evident after a pretreatment with 80 nM PC (Fig. 2).

The levels of lipid peroxidation were increased in cardiomyocytes exposed to 48 h hypoxia compared to normoxic cells. Pre-treatment with 80 nM and 2 μM compound **1** or compound **2**

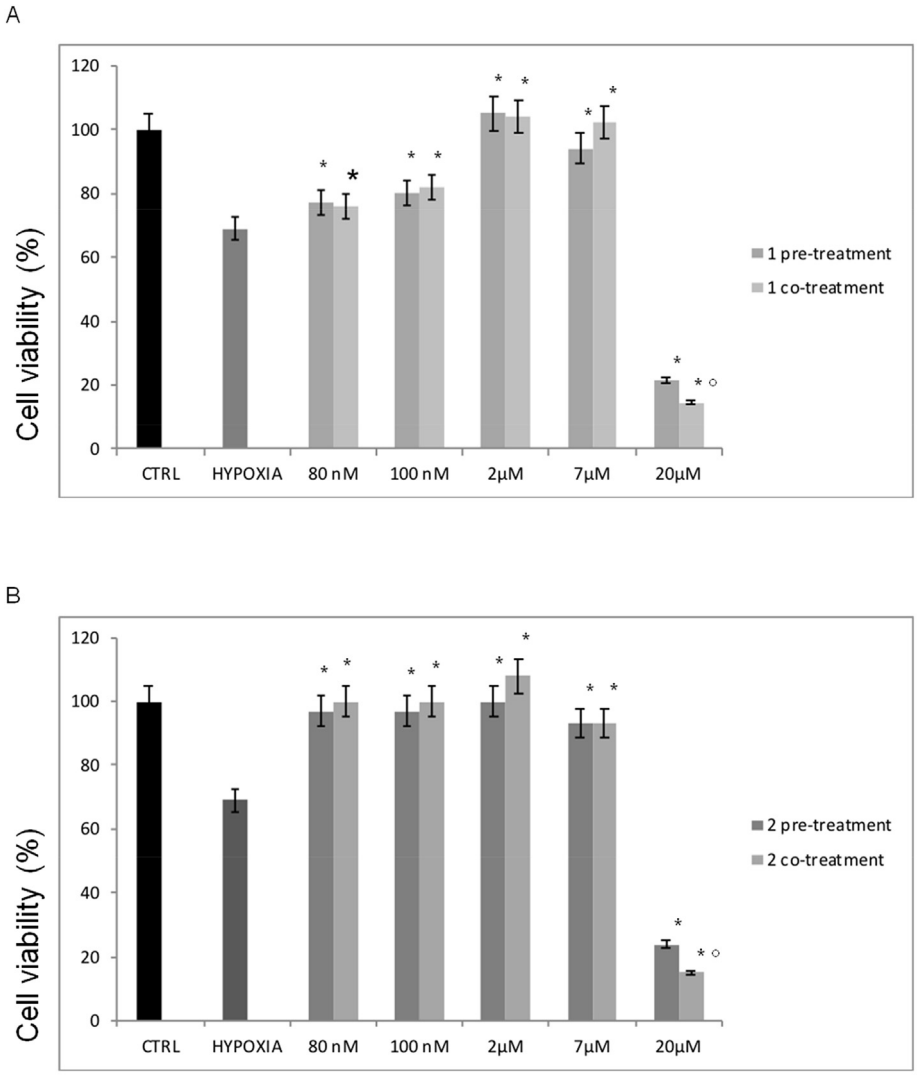


Fig. 1. Effects of resveratrol (**1**) and piceatannol (**2**) in pre-treatment (18 h) and co-treatment on the cell viability of H9c2 cells exposed to hypoxia for 48 h *p < 0.05 *vs* CTRL; °p < 0.05 *vs* 20 μM **1** or **2** pre-treatment.

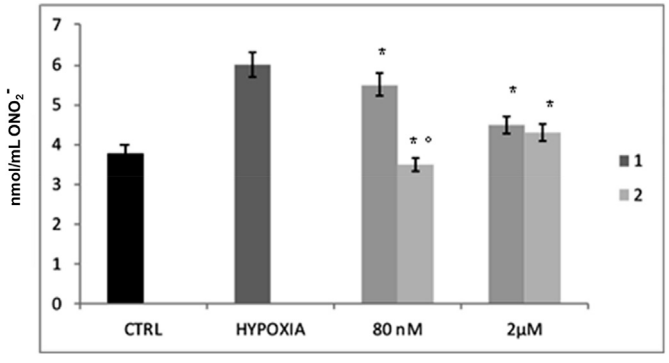


Fig. 2. Changes of released peroxynitrite in H9c2 cells under hypoxia treated with resveratrol (**1**) or picetannol (**2**) *p < 0.05 *vs* HYPOXIA ° p < 0.05 *vs* compound 1–80 nM.

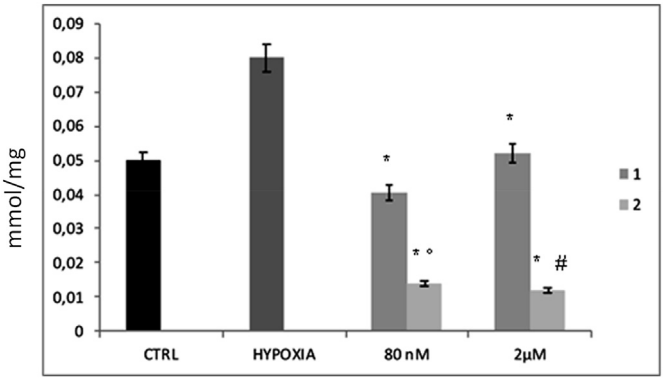


Fig. 3. Levels of thiobarbituric acid reactive substances (TBARS) in H9c2 cells under hypoxia exposed to a pre-treatment (18 h) with resveratrol (**1**) or picetannol (**2**). *p < 0.05 *vs* HYPOXIA ° p < 0.05 *vs* 1–80 nM #p < 0.05 *vs* 1–2 μM.

significantly and in a concentration-dependent manner reduced this effect (Fig. 3).

Several studies have demonstrated that the induction of MnSOD expression is related to the mechanism underlying the tolerance to hypoxia [60,61]. Forty-eight hours of hypoxia significantly induced a decrease of MnSOD protein expression and 18 h pre-treatment with 2 μM resveratrol (**1**) or piceatannol (**2**) significantly

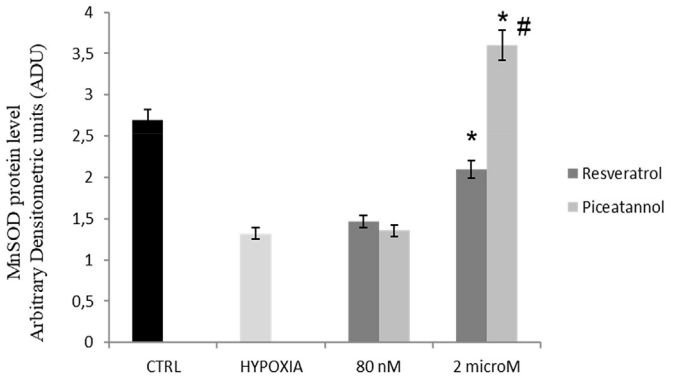


Fig. 4. Western blot analysis of MnSOD expression in H9c2 cells under hypoxia exposed to a pre-treatment (18 h) with resveratrol (**1**) or picetannol (**2**) at two concentrations (80 nM, 2 microM). *p < 0.05 *vs* HYPOXIA #p < 0.05 *vs* resveratrol 2 microM.

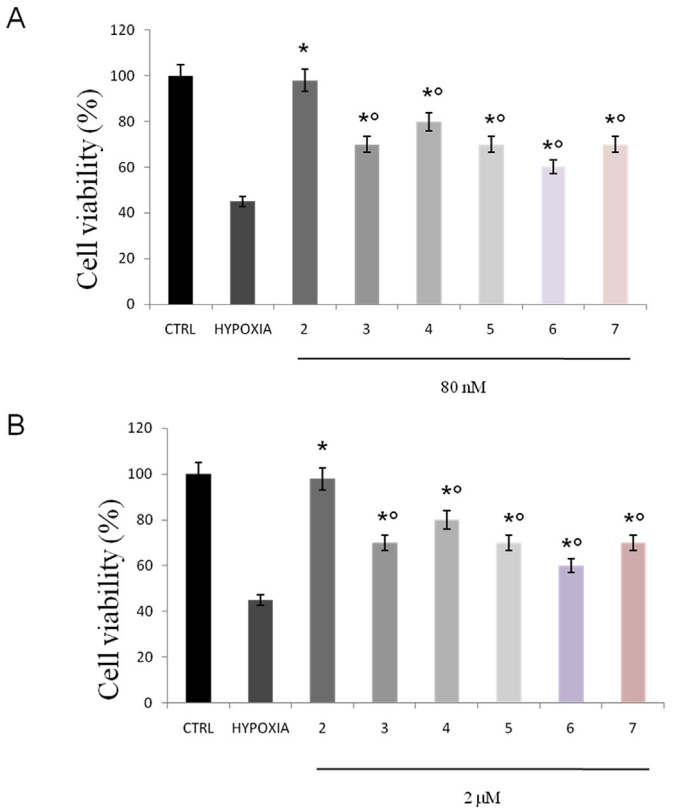


Fig. 5. Effects of a pre-treatment (18 h) with compounds **2**–**7** on the cell viability of H9c2 cells at 80 nM (A) and 2 μM (B) exposed to hypoxia. *p < 0.05 *vs* HYPOXIA ° p < 0.05 *vs* compound 2.

increased MnSOD protein levels compared to the concentration of 80 nM. This effect was more evident with piceatannol (**2**) treatment (Fig. 4).

2.3. Evaluation of protective activity against hypoxia-induced oxidative stress in H9c2 cells of synthetic analogues

All synthetic derivatives were tested in cardiomyocytes exposed to 48 h hypoxia (Fig. 5). The protective effect of piceatannol analogues against hypoxia-induced cell injury was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [62]. As shown in Fig. 5, the preliminary tests of analogues **3**–**7** at 80 nM revealed a recovery of cell viability of approx. 25–35% for all tested compounds.

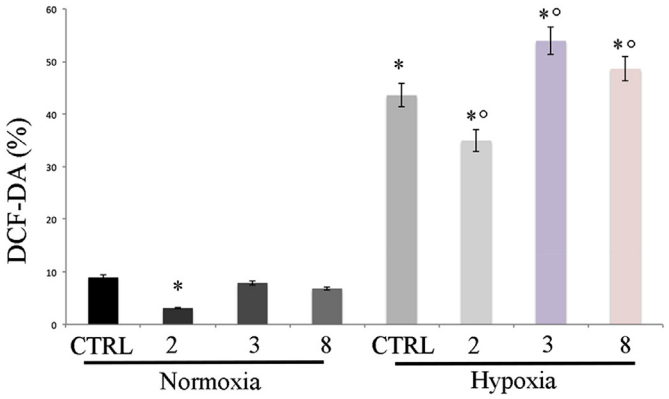


Fig. 6. The graph show the percentage of the DCF-DA positive cells after pre-treatment (18 h) with 2 μM of compounds **2**, **3** and **8** on H9c2 cells and then grown in normoxia and hypoxia condition.*p < 0.05 *vs* CTRL Normoxia ° p < 0.05 *vs* CTRL Hypoxia.

2.4. Evaluation of endogenous ROS after hypoxia-induced oxidative stress in H9c2 cells of synthetic analogues

Taking into account the interesting protective effect of piceatannol (**2**) we evaluated its ability to prevent reactive oxygen species (ROS) generation in cardiomyocytes exposed to hypoxia, in comparison with pterostilbene (**3**) and Zileuton (**8**). As shown in Fig. 6, a reduction of endogenous ROS formation, assessed using 2′,7′ –dichlorofluorescin diacetate (DCF-DA) assay, was revealed only for piceatannol (**2**) 38% *vs* 45% detected in hypoxia-induced cells.

2.5. Evaluation of effect on apoptosis after hypoxia-induced oxidative stress in H9c2 cells of synthetic analogues

Finally piceatannol (**2**) pterostilbene (**3**) and Zileuton (**8**) were tested with annexin V FITC to measure early apoptosis occurring during cytotoxicity in cardiomyocytes exposed to hypoxia. As shown in Fig. 7, the preliminary evaluation of analogues **2**, **3** and **8** at 2 μM revealed a reduction of early apoptosis only for derivate **3** (25% *vs* 60% detected in hypoxia-induced cells).

2.6. Evaluation of 5-LOX activity and structure-activity relationships

Analysis of test compounds as 5-LOX inhibitors was routinely carried out in two different test systems, a cell-free assay using isolated human recombinant 5-LOX and a cell-based assay using human neutrophils. The cell-free assay allows identifying compounds that directly interfere with 5-LOX catalytic activity, whereas the cell-based test system considers cellular regulatory aspects of 5-LOX product synthesis, and as such offers several points of attack of a given compound (e.g., inhibition of 5-LOX-activating protein (FLAP), interference with 5-LOX-activating lipid hydroperoxides, protein kinases or Ca^{2+} mobilization, and 5-LOX translocation/membrane association). The reference 5-LOX inhibitor *N*-[1-(1-benzothien-2-yl)ethyl]-*N*-hydroxyurea (zileuton) was used as the reference drug.

As shown in Table 2, all tested compounds caused significant inhibition of 5-LOX activity in both cell-based and cell-free assay, with IC_{50} values in the low micromolar range. Of note, the

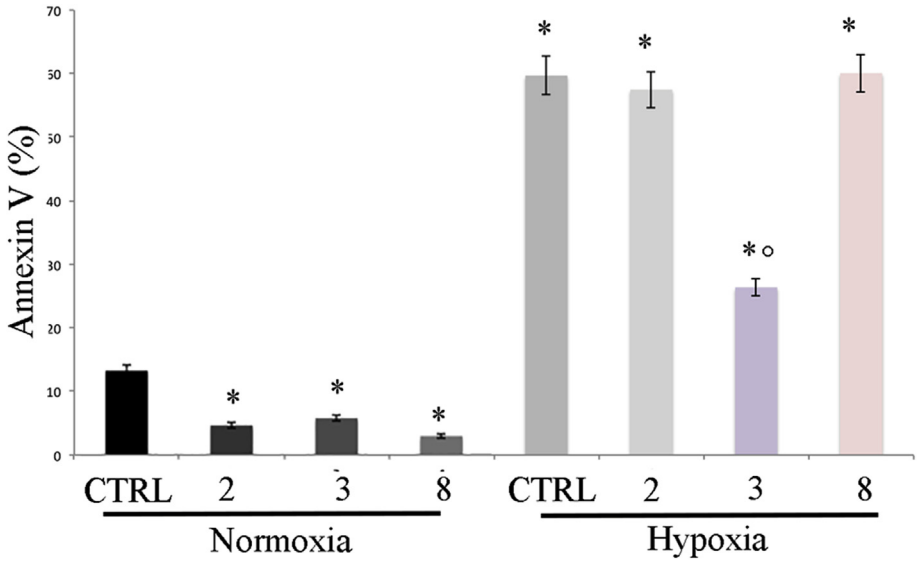
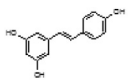
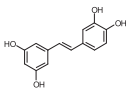
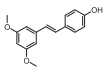
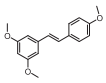
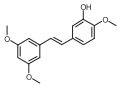
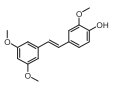
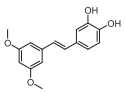


Fig. 7. The graph show the percentage of the Annexin-V positive cells after pre-treatment (18 h) with 2 μM of compounds **2**, **3** and **8** on H9c2 cells and then grown in normoxia and hypoxia condition. *p < 0.05 *vs* CTRL Normoxia ° p < 0.05 *vs* CTRL Hypoxia.

Table 2
Inhibition of 5-LOX activity of compounds 1–7 in a cell-based assay (intact PMNL) and in a cell-free assay.

Compound		Inhibition of 5-LOX activity (IC_{50} values [μM])	
		Intact	Cell-free
1		4.9 ± 0.6	63.4 ± 2.1%[a]
2		0.24 ± 0.09	1.1 ± 0.27
3		0.53 ± 0.12	3.91 ± 0.65
4		0.76 ± 0.19	3.62 ± 0.36
5		5.48 ± 1.99	5.49 ± 0.22
6		0.07 ± 0.01	0.78 ± 0.22
7		0.05 ± 0.01	0.32 ± 0.03
8	Zileuton	1.1 ± 0.40*	0.56 ± 0.1*

Data are given as mean ± S.E.M., n = 3–4. * [58].
[a] Remaining activity at 10 μM (%).

pterostilbene derivative **4** was more potent than resveratrol in both cell-free and cell-based experiments. Moreover, 3′-hydroxypterostilbene **7** (*trans*-3,5-dimethoxy-3′,4′-dihydroxystilbene), one of metabolites of pterostilbene and 3′-methoxypterostilbene (*trans*-3,5-dimethoxy-3′,4′-methoxystilbene) **6** were revealed as the most potent direct 5-LOX inhibitors with IC_{50} values of 0.32 and 0.78 μM in cell-free, and 50 and 70 nM in cell based assays, respectively.

It is interesting to note that when the 4′-OH group is converted into a methoxy group, the 5-LOX-inhibitory activity of the compounds radically decreased (**7** *vs* **5**) but this variation was not so evident with pterostilbene scaffold derivatives (**3** *vs* **4**) Moreover, the introduction of 3,5-dimethoxy motif at the A-phenyl ring of resveratrol, improved the 5-LOX inhibitory activity in both cell free and cell based evaluations (**7** *vs* **2**, **3** *vs* **1**). This is in agreement with

the recent findings, where pterostilbene, exhibited promising chemotherapeutic properties, may be due to the higher lipophilicity of pterostilbene scaffold, which may result in better permeability.

2.7. Docking studies with 5-LO

All of the seven molecules were docked into the crystal structure of human 5-LOX (PDB code: 3O8Y) [63]. After docking experiments, the best and the most energetically favorable pose of each ligand was selected. The protein-ligand complexes were analyzed to identify the most conserved interactions. The most active molecules compound **6** and compound **7** in the series ($IC_{50} = 0.07$ and 0.05 μM, respectively) are able to make strong interactions through hydrogen bonding with the side chains of both His372 and His367 as well as filling the hydrophobic region with 3,5-dimethoxy groups (Fig. 8F and G). Compounds **2**, **3** and **4** with IC_{50} values in the range of 0.24–0.76 μM are also positioned in the active site to establish similar H-bonding interactions with these His residues through their *p*-hydroxy or *p*-methoxy groups (Fig. 8B and C and D). The docking poses of the least potent compound **1** and compound **5** indicates that these compounds are not oriented appropriately to form these conserved H-bond interactions, which is in good agreement with the observed activity loss ($IC_{50} = 4.9$ and 5.48 μM, respectively).

3. Conclusion

Research and development of stilbene-based medicinal chemistry have become rapidly evolving with increasingly active compounds covering almost the whole range of therapeutic fields.

The results of the present work indicated that piceatannol (**2**) exerts a profound effect against hypoxia-induced injury for H9c2 cardiomyocytes through suppression of oxidative stress, while, with a direct evaluation of early apoptotic cells, pterostilbene (**3**) demonstrate suppressive effects on apoptosis. Moreover, piceatannol and synthetic pterostilbene derivatives showed significant activity in inhibiting 5-LOX in both cell-based and cell-free assay, with IC50 values in the low micromolar range. Additionally, the results indicate that minimal differences in the structure of polymethoxystilbenes can substantially affect both the inhibitory 5-LOX activity and the protection capability over cardiomyocyte injury. In this context, we highlight fast chemical methodologies for the synthesis of stilbene derivatives and outline the successful design of novel stilbene based hybrids as antiinflammatory and antioxidant compounds, that could be of interest in the development of targeted pharmaceutical approaches for limiting ischemic damage. This information may be useful in further design of stilbene-based molecules as new leads for the development of novel agents with clinical potential or as effective chemical probes. The results presented in this research cannot be generalized to animal models, but our new lead stilbene derivatives appear to be highly suitable for a posterior evaluation to dissect biological processes in the progression of myocardial tissue damage during ischemia. To this end, further studies are in progress to confirm the possibility of using these compounds in human therapies.

4. Experimental section

4.1. Chemistry

All reagents were analytical grade and purchased from Sigma–Aldrich (Milano, Italy). All microwave irradiation experiments were carried out in a Biotage® Initiator + Microwave synthesizer (Biotage, Sweden AB, Uppsala, Sweden). The reactions were carried out in 10 mL glass tubes, sealed with aluminium/Teflon crimp tops, which can be exposed to 300 °C and 30 bar internal pressure. After the irradiation period, the reaction vessel was cooled rapidly (60–120s) to ambient temperature by gas jet cooling. Flash chromatography was performed on Carlo Erba silica gel 60 (230–400 mesh; Carlo Erba, Milan, Italy). TLC was carried out using plates coated with silica gel 60F 254 nm purchased from Merck (Darmstadt, Germany). ^1H and ^{13}C NMR spectra were registered on a Brucker AC 300. Chemical shifts are reported in ppm. The abbreviations used are follows s, singlet; d, doublet; dd double doublet; bs, broad signal. MS spectrometry analysis ESI-MS was carried out on a Finnigan LCQDeca ion trap instrument.

4.1.1. General procedure for synthesis of compounds ***3,4,5,6,7***

The synthesis of piceatannol structural analogues was carried out under microwave irradiation by Suzuky-coupling conditions.

To a solution of *trans*-2-(3,5-dimethoxyphenyl)-vinylboronic acid pinacol ester (1.2 mmol) in EtOH abs. (2,5 mL), [1,1′ bis(diphenylphosphino)ferrocene]dichloropalladium (II) (0.005 mmol), K_2CO_3 anhydrous (2 mmol) and the corresponding brominated compound (1 mmol) were added. The reaction mixture was stirred and irradiated by microwave at 110 °C for 30 min. Then the reaction was filtered and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel using hexane/ethyl acetate (from 95:5 to 7:3) as eluent to give the corresponding derivatives.

4.1.2. 4-(3,5-dimethoxystyryl)phenol (***3***)

Yield: 43%. Rf: 0.4 in 8:2 hexane/ethyl acetate ^1H NMR ($CDCl_3$, 300 MHz): δ: 7.42 (d, 2H); 6.99 (dd, 2H); 6.85 (d, 2H); 6.68 (s, 2H); 6.42 (t, 1H); 3.86 (s, 6H). ^{13}C NMR ($CDCl_3$, 300 MHz): δ 55.4, 99.7, 104.4, 115.7, 126.6, 128.0, 128.7, 130.1, 139.7, 155.4, 160.9 ESI(*m*/*z*): 256.3 [$M^+ + 1$].

4.1.3. 1-(3,5-dimethoxystyryl)-4-methoxybenzene (***4***)

Yield: 46%. Rf: 0.4 in 9:1 hexane/ethyl acetate ^1H NMR ($CDCl_3$, 300 MHz): δ: 7.47 (d, 2H); 6.99 (dd, 2H); 6.91 (d, 2H); 6.68 (s, 2H); 6.40 (t, 1H); 3.86 (s, 9H). ^{13}C NMR ($CDCl_3$, 300 MHz): δ 55.4, 99.6, 104.3, 114.1, 126.6, 127.8, 128.7, 129.9, 139.7, 159.4, 160.9 ESI(*m*/*z*): 271.6 [$M^+ + 1$].

4.1.4. 5-(3,5-dimethoxystyryl)-2-methoxyphenol (***5***)

Yield: 44%. Rf: 0.6 in 6:4 hexane/ethyl acetate ^1H NMR ($CDCl_3$, 300 MHz): δ: 7.42 (d, 2H); 6.99 (dd, 2H); 6.85 (d, 2H); 6.68 (s, 2H); 6.42 (t, 1H); 3.86 (s, 6H). ^{13}C NMR ($CDCl_3$, 300 MHz): δ 55.4, 56.0, 60.4, 99.7, 104.4, 110.6, 111.9, 119.4, 127.1, 128.8, 130.9, 139.6, 145.8, 146.5, 160.9 ESI(*m*/*z*): 309.3 [$M^+ + 23$].

4.1.5. 4-(3,5-dimethoxystyryl)-2-methoxyphenol (***6***)

Yield: 42%. Rf: 0.7 in 7:3 hexane/ethyl acetate ^1H NMR (CD_3OD, 300 MHz): δ: 7.12 (s, 1H); 6.98 (d, 2H); 6.97 (dd, 2H); 6.79 (d, 2H); 6.36 (t, 1H); 3.89 (s, 3H); 3.79 (s, 3H). ^{13}C NMR (CD_3OD, 300 MHz): δ 55.4, 56.0, 60.4, 99.7, 104.4, 110.6, 111.9, 119.4, 127.1, 128.8, 130.9, 139.6, 145.8, 146.5, 160.9 ESI(*m*/*z*): 309.3 [$M^+ + 23$].

4.1.6. 4-(3,5-dimethoxystyryl)benzene-1,2-diol (***7***)

Yield: 43%. Rf: 0.6 in 6:4 hexane/ethyl acetate ^1H NMR (CD_3OD, 300 MHz): δ: 3.74 (s, 3H); 3.77 (s, 3H); 5,21 (s,-OH); 6.16 (s, 1H); 6,31 (d,2H); 6.56 (d,1H); 6.63 (s, 1H); 6, 76 (d, 1H); 6,88 (d, 2H). ^{13}C NMR (CD_3OD, 300 MHz): δ 55.9, 56.0, 101.4, 107.2, 114.1, 118.8, 121.7, 128.8, 128.9, 131.0, 136.6, 147.1, 147.3, 161.3, 161.9 ESI(*m*/*z*): 272.3 [$M^+ + 23$].

4.1.7. General procedure for synthesis of compounds ***2***

To a solution of **6** (1 mmol) in CH_2Cl_2 (4 mL), was added

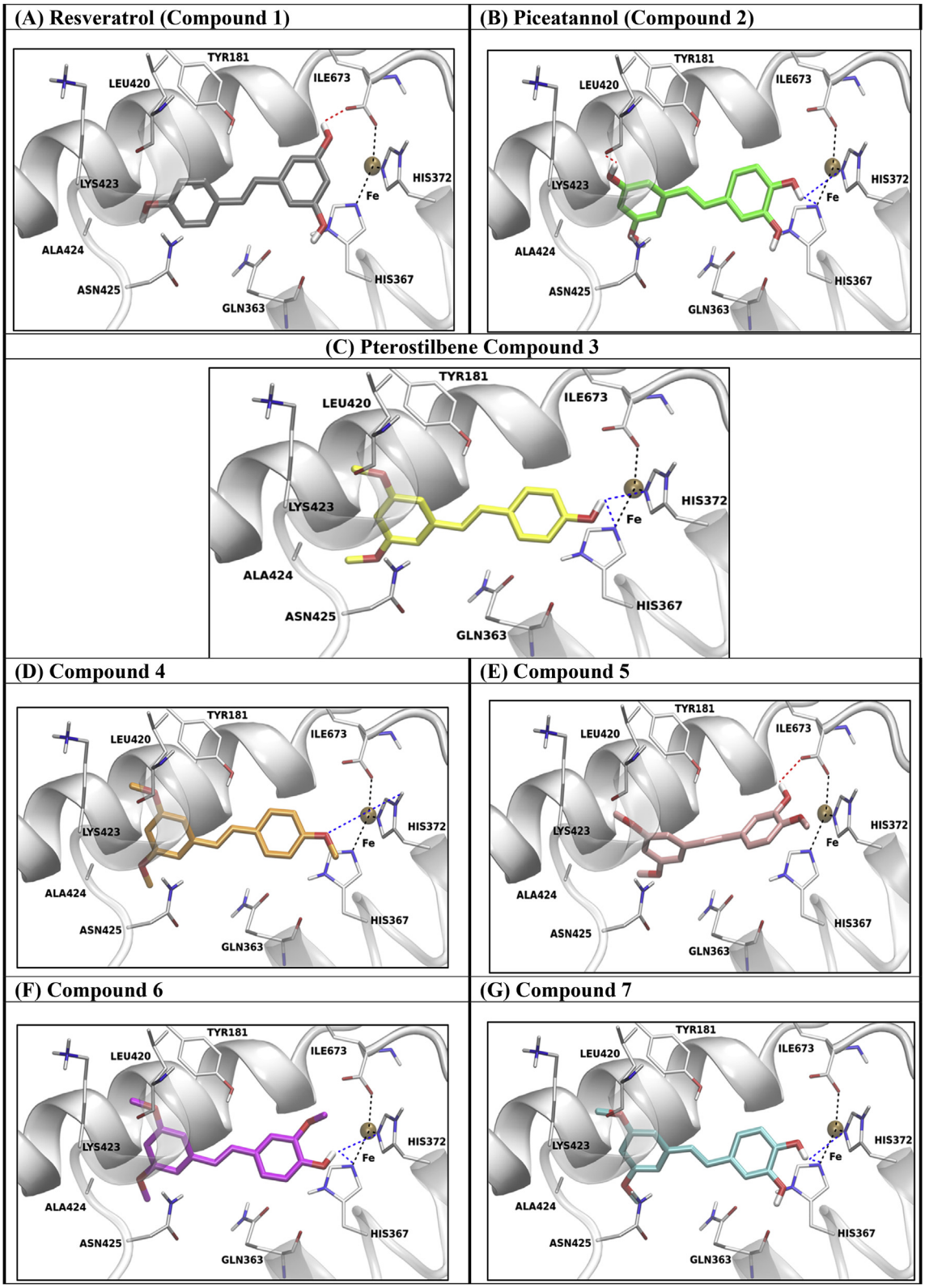


Fig. 8. A, B) Binding mode analysis of Resveratrol (1), Piceatannol (2) and Pterostilbene (3) depicted with interacting 5-LO (PDB code 3O8Y) residues. (D, E, F, G) Binding mode analysis of synthetic analogues 4–7. Main interactions are represented schematically with VMD 1.9.3. [74]

dropwise 1 mL of BBr_3 (solution 1 M in CH_2Cl_2) at temperature of 0 °C. The reaction mixture was stirred at room temperature over night and after was quenched by addition of 4 mL of water at 0 °C. The resulting mixture was stirred for 30 min at 0 °C. The aqueous layer was extracted with ethyl acetate and the organic extracts were combined, washed with brine, dried over Na_2SO_4 and concentrated by rotary evaporation. The crude product was purified by flash chromatography on silica gel using hexane/ethyl acetate (from 7:3 to 6:4) as eluent to obtain 62% of compound 2.^{1}H NMR data are in agreement with those reported in literature [35–37].

4.2. Biology

4.2.1. Cell culture

H9c2 rat cardiomyoblast cell line was purchased from the American Type Culture Collection (ATCC) and cultured in mixed growth medium (Dulbecco's modified Eagle's medium (DMEM) (Hyclone)) supplemented with 10% heat-inactivated FBS (Hyclone). Cells were kept in an incubator in an atmosphere of 5% CO_2 and 95% air at 37 °C and passaged at 1:3 ratio when they reached 80% confluence.

4.2.2. Hypoxic stress

For hypoxic stress 10^4 cells were seeded in 96-well plates and after 24 h incubation at 37 °C were washed once and stimulated with resveratrol and piceatannol at various concentrations (from 80 nM to 20 μM) at 37 °C both 18 h before the hypoxic stress that just before. After incubation, the cells were put in a humidified chamber sealed (Billups-Rothenburg, Del Mar, California) supplied with 5% carbon monoxide and 95% nitrogen for 48 h. Finally, MTT assay to test cell viability was performed. Using the same time points of treatment, before and just before the hypoxic stress, H9c2 cells were also treated with synthetic piceatannol derivatives (derivatives 3, 4, 5, 6 and 7 at 80 nM and 20 μM).

4.2.3. Cell proliferation assay

Cell proliferation was determined by 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Van Meerloo J). H9c2 cells (3×10^4 cells/mL) were seeded into the wells of 96-well culture plates as described above. MTT solution at 10% was added to each well and incubated for about 2 h. Then, excess medium was removed and 100 μL of a solution of 1 N hydrochloric acid at 10% in isopropanol to dissolve the formazan crystals. The mixture was shaken for about 20 min and the optical density in each well was measured using a microplate spectrophotometer (Microplate Reader Model 550, BIO-RAD, California, USA) at 570 nm. Triplicate experiments were performed for treatment with each concentration. The percentage cell viability (%) was calculated by comparison with a sample's corresponding control.

4.2.4. DCF-DA assay

The generation of reactive oxygen species (ROS) was monitored by the conversion of fluorogenic 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to highly fluorescent dichlorofluoresceindiacetate within cells by ROS.

The cells cultures were incubated for 30 min at RT with 2 μM DCFH-DA in PBS. Cells were washed with PBS acquired on a Guava® easyCyte™ flow cytometer (Millipore, Sigma) and analysed using easyCyte™ software.

4.2.5. Griess test

10^4 cells were seeded in 6-well plates and after 24 h incubation at 37 °C were washed once and stimulated with resveratrol and piceatannol (80 nM and 2 μM) at 37 °C 18 h and then were put in hypoxic chamber. At the end of the hypoxic stress the supernatants of each point were used to measure nitrite levels. NO is rapidly converted into stable end products nitrite and nitrate. Nitrite was measured by the Griess reaction as reported in literature. Briefly, 100 μL of supernatant was mixed with an equal volume of Griess reagent (0.5% sulfanilamide, 2.5% H_3PO_4, and 0.05% naphthylethylene diamine in H_2O) and incubated for 10 min at room temperature. Absorbance was assayed at 550 nm and compared with a standard curve obtained using sodium nitrite.

4.2.6. TBARS levels

10^4 cells were seeded in 6-well plates and after 24 h incubation at 37 °C were washed once and stimulated with resveratrol and piceatannol (80 nM and 2 μM) at 37 °C 18 h and then were put in hypoxic chamber.

At the end of the hypoxic stress cells were washed two times with cold PBS before lysis using 4 vol of modified RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.25% Na deoxycholate, 2% Triton-X100, 1 mM PMSF, 2 μM leupeptin). The protein samples were incubated with 0.5 ml of 20% acetic acid (pH 3.5) and 0.5 ml of 0.78% aqueous thiobarbituric acid solution. The mixture was heated to 95 °C for 45 min and centrifuged at 1600×*g* for 5 min. To quantify the amount of TBARS in the supernatant fractions, spectrophotometry was performed at an absorbance of 532 nm [27] and data were expressed as TBARS/serum protein in μM/μg. Data are presented as the average of triplicate measurements from duplicate experiments.

4.2.7. Western blot analysis

10^6 H9c2 cells were lysed by incubation on ice for 30 min with RIPA lysis buffer and 10 μL/mL leupeptin, 5 μL/mL aprotinin, 1 μmol/L pepstatin, and 10 mmol/L DTT were added before use. After centrifugation, the supernatant was collected, and the protein concentration was measured using a commercial kit (Bio-Rad Laboratories, Milan, Italy) according to the manufacturer's instructions. 80 μg of protein samples were run on 12% SDS–PAGE gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Milan, Italy). Membranes were blocked for 1 h at room temperature with 5% milk in tris buffer saline (TBS) with 0.1% Tween 20 (T-TBS), followed by incubation at 4 °C overnight with primary antibodies against manganese superoxide dismutase (MnSOD) (Merck Millipore, Germany) Membranes were then washed three times with 0.1% T-TBS solution, and incubated for 1 h at room temperature with a secondary antibody goat anti-rabbit IgG-HRP or goat anti-mouse IgG-HRP respectively, according to the primary antibodies data sheet (Santa Cruz Biotechnology, Milan, Italy). GAPDH antibody (Santa Cruz Biotechnology, Milan, Italy) was used as an internal standard. The immunoreactive bands were visualized using an enhanced chemiluminescence system (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce, Rockford, USA). The protein bands were scanned and quantitated with Gel Doc-2000 (Bio-Rad, Milan, Italy).

4.2.8. Apoptosis assay

Apoptotic cells were detected using fluorescein-conjugated annexin V with a Nexin® kit on a Guava® easyCyte™ (Millipore Sigma, Burlington, MA, USA) flow cytometer following the manufacturer's instructions. Samples were acquired on a Guava® easyCyte™ flow cytometer (Millipore, Sigma) and analysed using easyCyte™ software.

4.2.9. Evaluation of 5-LOX activity

4.2.9.1. Determination of 5-LOX product formation in cell-based assays. Polymorphonuclear leukocytes (PMNL) were isolated from human blood of adult healthy volunteers, with consent, obtained from the Institute of Transfusion Medicine, University Hospital

Jena, as described [64]. PMNL (5×10^6 in 1 ml PBS plus 1 mg/ml glucose and 1 mM $CaCl_2$) were preincubated with the test compounds (15 min, 37 °C) and 5-LOX product formation was started by addition of 2.5 μM A23187 [65]. After 10 min at 37 °C, the reaction was stopped with 1 ml of methanol. Formed 5-LOX metabolites were extracted and analyzed by HPLC as described [66]. 5-LOX product formation is expressed as ng of 5-LOX products per 10^6 cells, which includes LTB_4 and its all-trans isomers, 5(S),12(S)-di-hydroxy-6,10-trans-8,14-cis-eicosatetraenoic acid (5(S),12(S)-DiHETE), and 5(S)- hydro(pero)xy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-H(p)ETE). Cysteinyl LTs C_4, D_4 and E_4 were not detected, and oxidation products of LTB_4 were not determined.

4.2.9.2. Expression and purification of human recombinant 5-LO from E. coli, and determination of5-LOX activity in cell-free systems. *E.coli* BL21 was transformed with pT3-5LO plasmid, human recombinant 5-LOX protein was expressed at 37 °C, purified as described [67], and immediately used for 5-LOX activity assays. 5-LOX (0.5 μg) was diluted with PBS/EDTA and pre-incubated with test compounds. After 15 min at 4 °C, samples were pre-warmed for 30 s at 37 °C, and 2 mM $CaCl_2$ plus 20 μM arachidonic acid were added to start 5-LOX product formation. After 10 min at 37 °C formed metabolites were analyzed by HPLC as described for intact cells above.

4.3. Docking study

The binding modes and interaction analysis of all reported derivatives for 5-LOX were studied by applying docking procedure as previously published [68,69]. Shortly, the PDB entry for 5-LOX (PDB code: 3O8Y) [63] was first submitted to the Protein Preparation Wizard [70] protocol of the Schrödinger Suite 2017-1for force field (OPLS2005) parameterization and active site relaxation procedures [71]. Next, ligands were prepared by the LigPrep to assign the protonation states and atom types of the molecule with OPLS2005 forcefield [72]. The grid generation and docking runs were done in single precision mode (GlideScore SP) by Glide 7.4. [73] The best ranking pose was visualized with VMD 1.9.3. [74].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2019.07.033.

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