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Antioxidant activity of benzoxazolinonic and benzothiazolinonic derivatives in the LDL oxidation model

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ABSTRACT

A series of benzazolone compounds were synthesized utilizing benzoxazolinonic and benzothiazolinonic heterocycles as the building unit. The antioxidant activity of these compounds was determined by inhibition of lipid peroxidation. The oxidation of LDL was induced in the presence of $CuSO_4$ or 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH). The protective action of these compounds against the cytotoxicity was evaluated with lactate dehydrogenase (LDH) activity in bovine aortic endothelial cells (BAECs) and cellular vitality by measuring mitochondrial activity in the presence of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The best antioxidant activities were observed for phenolic compounds **13** and **14b** with ratio $R = 2.5$, 3.2 (5 μM). Both of these test substances increased the cell viability significantly as indicated by MTT assay and LDH release assay.

1. Introduction

The pineal gland secretes a number of pineal indoles including melatonin. Melatonin (*N*-acetyl-5-methoxytryptamine), a highly conserved naturally occurring molecule, is a pineal hormone that regulates circadian rhythms and also protects against oxidative stress-induced tissue damage, lipid peroxidation, and apoptosis.[1,2] Antioxidant actions of melatonin are observed at different levels including attenuation of radical formation, which is also referred to as radical avoidance. Although melatonin efficiently interacts with various reactive oxygen species (ROS) and reactive nitrogen species as well as with organic radicals, it also upregulates antioxidant enzymes and downregulates pro-oxidant enzymes.[3–6]

Cardiovascular diseases (coronary heart disease, stroke, etc.), depending on atherosclerosis development, remain the major cause of death in most developed countries. Atherosclerosis is a chronic vascular disease in which inflammation and oxidative stress are commonly implicated as major causative factors. Early stages of plaque development involve endothelial activation induced by inflammatory cytokines, oxidized low-density lipoprotein (ox-LDL), and/or changes in endothelial shear stress. It has been clearly demonstrated that hypercholesterolemia secondary to high LDL plasma levels and subsequent oxidation of these lipoproteins are the major causes of atherosclerosis in humans and different animal models.[7] LDL particles which accumulate in the plasma infiltrate the intimal space of the arteries and are oxidized by free radicals. There is considerable experimental evidence to show that several different antioxidant compounds given at high pharmacological doses are effective in decreasing both LDL oxidation and atherogenesis in animals.[8] In humans, supplementation with antioxidants combined at physiological doses is incapable of inhibiting coronary heart disease in primary prevention.[9] Antioxidants would have to be given at high pharmacological doses in humans to inhibit ex vivo Cu^{2+}-induced LDL oxidation.[10]

Duell [11] tested the capacity of melatonin in inhibiting the oxidation of LDL in a standardized in vitro system. They showed that melatonin had no antioxidant activity at physiologic concentrations and only moderate antioxidant activity at concentrations that were 4–6 orders of magnitude greater than peak physiologic concentrations. Therefore it seems interesting to study the free radical scavenging and antioxidant activity of melatonin derivatives that act as strong antioxidants and that could not act on circadian rhythm.

The potent endogenous antioxidant melatonin possesses several analogs or metabolites, and several effects attributable to melatonin have been suggested to be mediated by these metabolites.[12] Pinoline (6-methoxy-1,2,3,4-tetrahydro-β-carboline), a minor cyclic metabolite of melatonin[13], has been shown to inhibit lipid peroxidation in different model systems.[14,15] Starting from pinoline, previous research were realized in our laboratory to lead a β-carboline compound GWC22.[16] This compound has been shown to display better antioxidant properties than melatonin toward peroxyl-induced peroxidation of a linoleate model system.[17–19]

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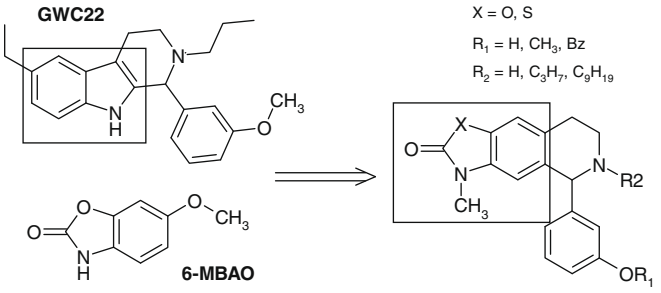


Figure 1. Design of benzoxazolinonic and benzothiazolinonic derivatives.

6-Methoxy-2-benzoxazolone (6-MBAO) was described in the literature as exhibiting an antioxidative potency similar to that of melatonin.[20] Both the structures, GWC22 and 6-methoxy-2-benzoxazolinone, allowed to design a new family of benzoxazolinonic and benzothiazolinonic derivatives with potent antioxidant activities (Fig. 1). Benzoxazolone and the sulfur bioisoster, that is, benzothiazolone, were frequently used for medicinal chemistry.[21] In continuation with previous studies on the biologically active β-carboline derivatives of melatonin, this work aimed to study the possible protective effect of novel benzazolone derivatives.

In this family, we have modulated the nature of the heterocycle with benzoxazolone (X = O) and benzothiazolone (X = S), the length of the linear chain on the nitrogen atom of compounds with propyl and nonyl groups. Various substituents (R_1) were introduced on the 3-position of the phenyl ring with R_1 as hydrogen atom, methyl, or benzyl groups. Then, the oxazolinonic ring of the benzoxazolone heterocycle was open in basic media to give the corresponding aminophenol derivatives.

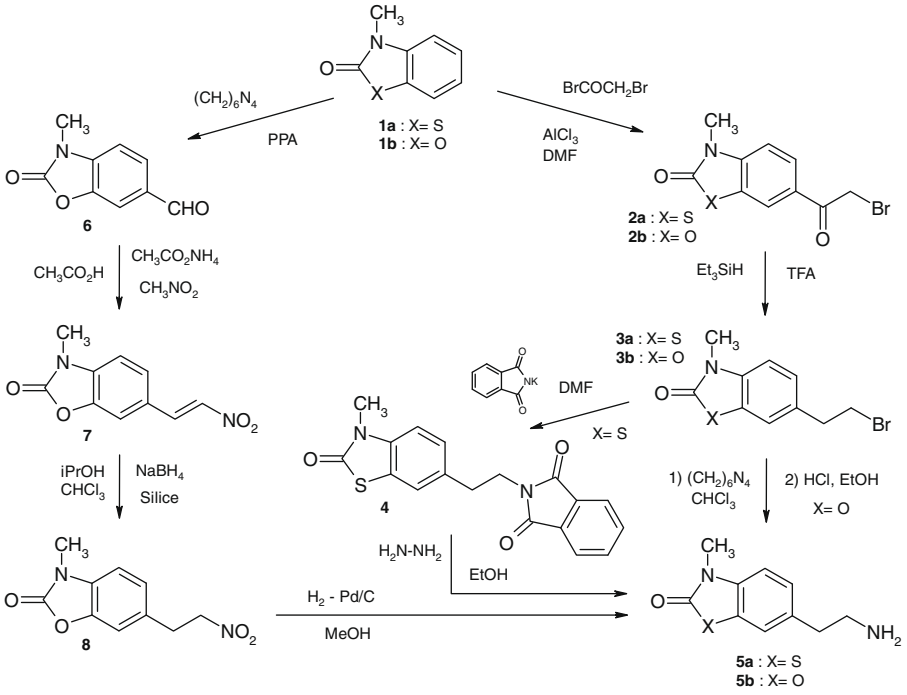
2. Results and discussion

2.1. Chemistry

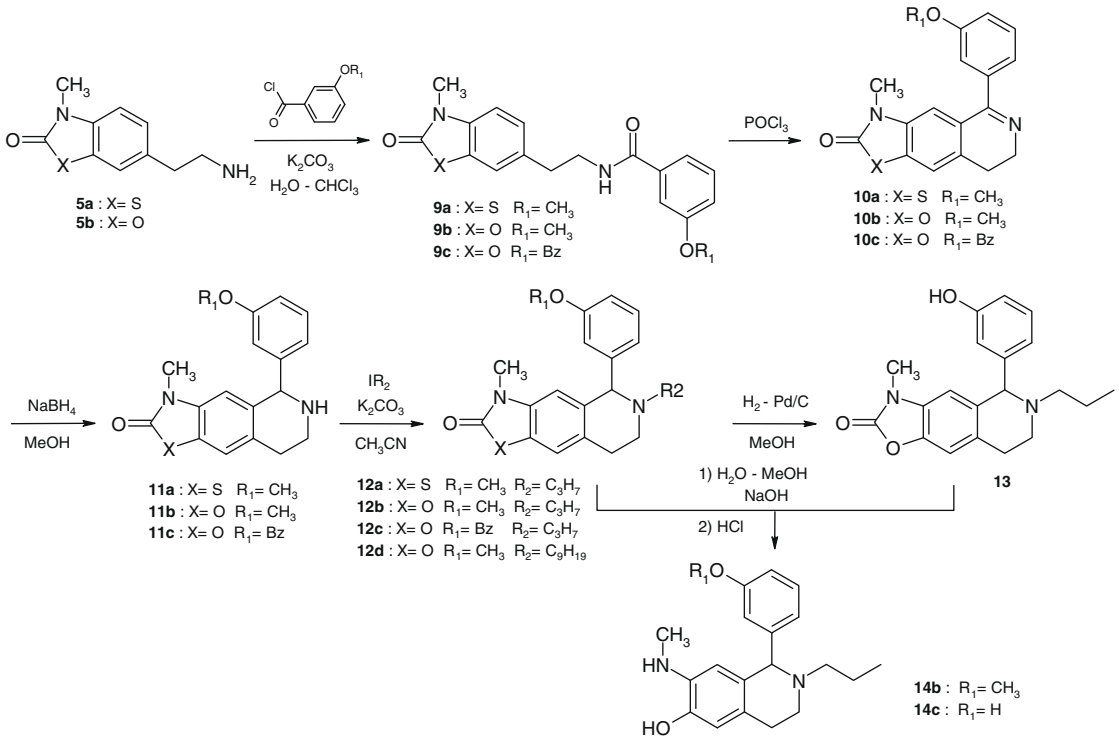
Scheme 1 illustrates the procedures used for the synthesis of the required primary amines 5**a**–**b** starting from 3-methyl-2(*3H*)-benzazolones (**1a** and **1b**). From 3-methyl-2(*3H*)-benzazolone (**1a** and **1b**), 6-bromoethyl-3-methyl-2(*3H*)-benzazolone (**3a** and **3b**) were obtained by a Friedel–Craft acid-catalyzed acylation[22,23] followed by a reduction of the ketone carbonyl group.[23,24] While compound **5b** was obtained via a Delepine condensation[25] of the bromoethyl derivative (**3b**) with hexamethylenetetramine in good yield (72%), application of this procedure to access compound **5a** was unsuccessful. Derivative **5a** was obtained in two steps with good yield (74%), starting from compound **3a** in a Gabriel reaction, the phthalimidoethyl derivative (**4**) was obtained in dimethylformamide with phthalimide potassium salt, then a reaction of hydrazinolysis in ethanol afforded compound **5a**.[26] This methodology could not be applied to benzoxazolone derivative (**3b**), the hydrazinolysis also opens the oxazole ring.

Another way was attempted in four steps to synthesize compound **5b**. 3-Methyl-2(*3H*)-benzoxazolone was formylated using hexamethylenetetramine in polyphosphoric acid (PPA),[27] to afford compound **6** . The formyl derivative **6** was condensed with nitromethane in the presence of a catalytic amount of ammonium acetate to afford the expected nitrovinyl product **7** by a Henry reaction.[28,29] Reduction of the α,β-unsaturated nitroalkene **7** to the desired saturated compound **5b** was performed with sodium borohydride in a mixture of chloroform and isopropanol in the presence of silica gel, followed by hydrogenation over Pd/C. This way gave low yield of 29% while compound **5b** was obtained using the first way in three steps with 47% yield.

Scheme 2 illustrates the procedures used for the synthesis of benzazolinonic analogs of GWC22. Under Schotten–Baumann reaction conditions, aminoethyl compounds **5a**–**b** were reacted with phenyl acyl chloride derivatives to furnish the expected amide derivatives **9a**–**c**. Classical Bischler–Napieralski cyclization afforded, in the presence of phosphorous oxychloride, compounds **10a**–**c**, which were reduced in methanol by sodium borohydride to give derivatives **11a**–**c**. Attempts using Pictet–Spengler reaction[30] in CH_2Cl_2 with TFA, compound **5a** or **5b,** and the desired benzaldehyde, to furnish compounds **11a**–**c** were unsuccessful. Alkylation of compounds **11a**–**c** by *n*-propyl or *n*-nonyl iodide, in acetonitrile with potassium carbonate, provided the corresponding



Scheme 1. Synthesis of 6-ethylamino-3-methyl-2(*3H*)-benzazolones (**5a** and **5b**).



Scheme 2. Synthesis of final compounds **12a–d**, **13,** and **14b–c**.

tertiary amines (**12a–d**). Deprotection reaction of the benzyl group of compound **12c**, in methanol with Pd/C under atmospheric pressure of hydrogen, gave compound **13** with phenolic group. Compounds **12b** and **13**, in a solution of water/methanol with sodium hydroxide, afforded after acidification the aminophenolic compounds **14b** and **14c**.

2.2. Antioxidant activity

The oxidation of LDL was determined by measuring conjugated diene formation at 234 nm for 480 min. To eliminate the possible role of Cu-chelation, oxidation was induced by water-soluble 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), which generates free radical, by its spontaneous thermal decomposition.

In the presence of $CuSO_4$ or AAPH, the OD_{234} curve versus time corresponds to the typical one described by Esterbauer,[31] including the lag, propagation, and decomposition phases.

Without Cu^{2+} or AAPH, the kinetics of diene conjugate formation was slower, and the final concentration was dramatically lower after 24 h of oxidation. The lag phase for the initiation of LDL oxidation in the presence of copper or AAPH was about 100 min. The capacity of derivatives to reduce or inhibit copper or AAPH-initiated LDL oxidation was tested and the kinetics of LDL oxidation led us to calculate the ratio *R* (Table 1).

R represents the ratio between the length of the lag phase of the oxidative procedure of LDL with different compounds and the length of the one without compound (control).

Addition of 1–100 μM of tested compounds to the LDL solution induced a dose-dependent increase in the lag phase of conjugated diene formation (data not shown). Results at 5 M are represented in Table 1.

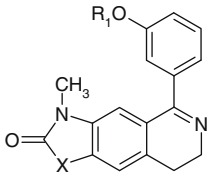
Excepted for compounds **10b** and **11b,** ($R = 1$ for 5 M Cu^{2+}) the study confirms that the derivatives reduce copper-induced LDL oxidation (for 5 M Cu^{2+}). The most antioxidant products in a copper system were tested in AAPH oxidation system. In the presence of the tested molecules there was a strong correlation in the lag phase duration between these two oxidative procedures of LDL. Therefore, the free radical scavenging mechanism contributes toward the inhibitory action of these compounds.

All compounds, designed starting from derivatives GWC22 and 6-MBAO, showed antioxidant activities. Indeed, for compounds **10b** and **11b** an antioxidant effect is observed at 5 μM ($R = 1.8$), the antioxidant activity is lower than that of the other compounds synthesized but higher than that of 6-methoxy-2-benzoxazolone and melatonine[21] ($R = 1.5$ for 100 μM). Benzothiazolone compounds (**10a**, **11a**, and **12a**, respectively, with $R = 2.5$, 2.1, and 2.1 for 5 μM) have more antioxidant activity than derivative GWC22 ($R = 1.6$ for 5 μM) and their benzoxazolone analogs (**10b**, **11b**, **12b**, respectively, with $R = 1$, 1, 1.5 for 5 μM) which could be explained by an improved lipophilicity with the sulfur derivatives (**10a**, **11a**, **12a**). Substitution with benzyl group induced the best results when we compared compounds **10b**, **11b** ($R = 1$ for 5 μM) with **10c**, **11c** ($R = 1.5$ for 5 μM) and compound **12b** ($R = 1.5$ for 5 μM) with **12c** ($R = 2.2$ for 5 μM), this compound **12c** showed the best antioxidant activities in this benzoxazolone series superior to GWC22. In previous paper, the authors demonstrated the relationship between the chain length (which influences the Log *P*) and the antioxidative activity.[32,33] In the benzoxazolone series we have modulated the chain length at the N-2 position with derivatives **12b** and **12d**, but we observed similar antioxidant activities ($R = 1.5$ and 1.7 for 5 μM) for these compounds. Phenolic derivatives were well described to enhance antioxidant activities, so we synthesized phenolic compound **13**, by a debenzylation reaction of compound **12c**, which displays good antioxidant activities with a ratio $R = 2.5$ for 5 μM. Starting from derivatives **12b** and **13**, the ring opening of the oxazole led to aminophenolic derivatives **14b** and **14c**. Compound **14c** due to the color at 234 nm could not be tested for the antioxidant activities, while **14b** exhibited the best antioxidant activity with a ratio $R = 3.2$ for 5 μM of the series.

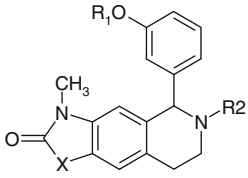
2.3. Cytotoxicity assays

In vivo low-density protein (LDL) oxidation is a progressive phenomenon leading to the presence of minimally and highly

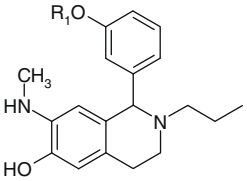
Table 1
Antioxidant activities and cytotoxicity assays of compounds **10a–c**, **11a–c**, **12a–d**, **13,** and **14b,c**



10a-c



11a-c, 12a-d, 13



14b-c

Compd	R_1	R_2	Activity (R)				LDH release in %
			100 μM (Cu^{2+})	10 μM (Cu^{2+})	5 μM (Cu^{2+})	5 μM (AAPH)	
Melatonin	—	—	1.5	—	—	—	—
6-MBOA	—	—	1.5	—	—	—	—
GWC 22	—	—	>5	>5	1.6	1.5	15.32 ± 1.56
10a	CH_3	—	>5	>5	2.5	2.5	14.12 ± 1.58
10b	CH_3	—	>5	1.8	1	—	
10c	Bz	—	>5	3.2	1.5	1.4	17.2 ± 1.85
11a	CH_3	—	>5	>5	2.1	2	14.50 ± 1.62
11b	CH_3	—	>5	1.8	1	—	
11c	Bz	—	>5	3.3	1.5	1.6	16.50 ± 1.75
12a	CH_3	C_3H_7	>5	>5	2.1	1.9	14.60 ± 1.65
12b	CH_3	C_3H_7	>5	3.3	1.5	—	
12c	Bz	C_3H_7	>5	>5	2.2	2.2	13.85 ± 1.48
12d	CH_3	C_9H_{19}	>5	3.8	1.7	—	
13	H	C_3H_7	>5	>5	2.5	2.4	13.50 ± 1.45
14b	CH_3	C_3H_7	>5	>5	3.2	3.3	13.20 ± 1.42
14c	H	C_3H_7	—	—	—	—	14.10 ± 1.51

R: Ratio between the length of the lag phase of the oxidative procedure of LDL with different compounds and the length of the one without compound (control).
LDH activity was expressed as the percentage of total LDH cellular release following the addition of Triton X-100 (final concentration of 0.1% v/v in ethanol). The cells were pretreated for 24 h with 10 μM drugs, then for 16 h with the same concentration of drugs and 100 μg/mL of mo-LDL. The LDH release in the presence of mo-LDL alone is 22.76 ± 1.53%.

oxidized LDLs in the subendothelial arterial space. Oxidized LDLs have been reported to be cytotoxic against endothelial cells. Furman[34] in 1999 showed the differential toxicities of air (mO-LDL) or Copper-Oxidized LDLs (Cu) toward endothelial cells. Both the morphological aspect of the cells themselves and LDH test revealed that mO-LDLs had cytotoxicity with up to 8 h of oxidation.

The protective effect of melatonin derivatives against cytotoxics mO-LDL was studied in cultured bovine aortic endothelial cells (BAECs). This model of primary culture cells is commonly used in our laboratory.

Normal LDL (100 μg protein/ml) in cellular medium induced minor cellular shape changes and after 16 h of incubation most cells remained attached to the bottom of culture wells. Incubation with 100 μg protein/ml mO-LDL for 16 h resulted in major morphological cellular changes: the initial visual change consisted in cell contraction, with the appearance of cytoplasmic ramification, then some cells rounded up and detached from the bottom of the wells. After treatment with GWC22 or melatonin derivatives the number of detached cells decreased and fixed cells were less shrunk.

In this study, incubation of cultured endothelial cells with mO-LDL (100 μg protein/ml) for 16 h induced toxicity, characterized by cellular morphological changes, an increase in cellular LDH release, and a decrease in MTT activity. When we add benzazolinones derivatives to the medium there is a decrease of the cellular LDH release and an increase of MTT reactivity. Cytotoxicity was quantified using LDH release. LDH release increased when cells were treated with mO-LDL in comparison with normal LDL (22.76 ± 1.53% vs 11.07 ± 2.8, respectively). In the presence of normal LDL the drugs had no significant effect on cellular LDH release, while in the presence of mO-LDL, GWC 22 and other product derivatives significantly reduced LDH activity.

In summary our results show that the most antioxidant products are also the less cytotoxics. For example, for compounds **10c** and **11c** (R = 1.5 for 5 μM), LDH release reaches 17.2% and 16.5%, respectively. We observed the best antioxidant activities (R = 2.5 and 3.2, respectively for 5 μM) and the lower LDH release 13.5% and 13.2%, respectively, for compounds **13** and **14b**. Cellular vitality was determined by measuring mitochondrial activity in the presence of MTT:(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). After 16 h of incubation with mO-LDL alone MTT reactivity is about 60%. With the different derivatives synthesized, all the activities reach nearly 90 ± 1% without differences between them.

These biological properties look important in term of prevention of cardiovascular diseases[33,35] but the capacity of molecules to inhibit atherogenic lipoprotein oxidation in vitro offers no prediction of their capacity to inhibit in vivo atherosclerosis development. Tailleux[18,36] rated that the feeding of hypercholesterolemic mice on an atherogenic diet supplemented with melatonin highly increases the surface of atherosclerotic lesions in aorta and the sensitivity of atherogenic lipoprotein to ex vivo oxidation even though high melatonin doses inhibit lipoprotein oxidation in vitro. A melatonin-related compound (DTBHB: *N*-[2-(5-methoxy-1*H*-indol-3-yl)ethyl]-3,5-di-*tert*-butyl-4-hydroxybenzamide) has been reported to strongly inhibit lipid peroxidation in vitro and considerably increase the sensitivity of atherogenic lipoproteins to ex vivo oxidation but did not modify atherosclerotic lesion development in mice.

The studies in vivo are necessary to confirm the cardiovascular protective effects of the benzazolinone derivatives.

3. Conclusion

The synthesized compounds displayed interesting antioxidant activity superior to those of melatonin and 6-methoxy-2-benzoxazolinone. Compounds in the benzothiazolone series gave higher

antioxidant activities than the benzoxazolone series. The modulation with a benzyloxy group on the phenyl ring was important for antioxidant activity (**10c**, **11c**, and **12c**), identically for compounds **13** and **14b** with the presence of phenolic group.

The oxidative modification of LDL has been alleged to play an important role in the development of human atherosclerosis. Thus, protecting LDL from oxidation by compounds such as melatonin derivatives may be an effective strategy to delay or prevent the progression of the disease. The toxicity of mO-LDL exposed to melatonin derivatives is weaker in terms of LDH release. Because oxidation of LDL is implicated in the pathogenesis of atherosclerosis, the enhancement of LDL resistance to oxidation is one of the models used by many researchers to investigate the efficacy of new drugs as antioxidants against radicals generated in the lipophilic phase. The remarkable capacity of these derivatives to function in vitro as antioxidants has been demonstrated in this study. They exhibit high antioxidant protective activity against peroxidations induced by metal ions or peroxyl radical. Furthermore, they do not chelate copper, suggesting that they may exert their antioxidant effect on lipid peroxidation primarily by scavenging free radicals. The capacity of molecules to inhibit atherogenic lipoprotein oxidation in vitro offers no prediction of their capacity to inhibit in vivo atherosclerosis development. To demonstrate beneficial effects on atherosclerosis we need to make further experiments in vivo.

4. Experimental

Compounds were purified on a glass column using Merck Silica Gel 60 (230–400 mesh). Melting points were determined by a Büchi 510 capillary apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker AVANCE 300 spectrometer and chemical shifts are in ppm with TMS as internal standard. Mass spectra were recorded on a quadripolar Finnigan Mat SSQ 710 instrument.

4.1. 3-Methyl-6-(2-nitrovinyl)-2(*3H*)-benzoxazolone (7)

To 35 mL of acetic acid, 6-formyl-3-methyl-2(3*H*)-benzoxazolone (4 g, 22 mmol), ammonium acetate (3.83 g, 49 mmol) and nitromethane (18 mL, 330 mmol) were added. The reaction mixture was refluxed for 2 days. Water (100 mL) was introduced and the resulting precipitate was filtered and recrystallized in methanol. Yield 72%; mp 195–197 °C; ^{1}H NMR (300 MHz, $CDCl_3$), δ = 3.50 (s, 3H, NCH_3), 7.08 (d, 1H, H_4, J = 8.2 Hz), 7.43 (d, 1H, H_7, J = 1.5 Hz), 7.46 (dd, 1H, H_5, J = 8.2 Hz, J_{5-7} = 1.5 Hz), 7.60 (d, 1H, CH, J = 13.5 Hz), 8.06 (d, 1H, CH, J = 13.5 Hz); Mass *m/z* 222.2 (M^++1).

4.2. 3-Methyl-6-(2-nitroethyl)-2(*3H*)-benzoxazolone (8)

To a mixture of chloroform/propan-2-ol (30/5, v/v), 3-methyl-6-(2-nitrovinyl)-2(3*H*)-benzoxazolone (1.1 g, 4.8 mmol), silica (1.5 g) and sodium borohydride (0.43 g, 12.1 mmol) were added. The reaction mixture was stirred for 1 day at room temperature, and the reaction was stopped by dropwise addition of acetic acid (5 mL). The reaction mixture was stirred for an additional 15 min. The insoluble material was filtered and washed with dichloromethane, and then the solvent was removed in vacuo. The resulting precipitate was recrystallized in absolute ethanol. Yield 68%; mp 134–136 °C; ^{1}H NMR (300 MHz, $CDCl_3$), δ = 3.25 (t, 2H, CH_2, J = 7.1 Hz), 3.30 (s, 3H, NCH_3), 4.85 (t, 2H, NCH_2, J = 7.1 Hz), 7.12 (d, 1H, H_4, J = 8.2 Hz), 7.18 (d, 1H, H_5, J = 8.2 Hz), 7.31 (s, 1H, H_7); Mass *m/z* 224.1 (M^++1).

4.3. 3-Methyl-6-(2-aminoethyl)-2(*3H*)-benzoxazolone hydrochloride (5b)

To 20 mL of methanol, compound **8** (1.1 g, 4.8 mmol) and Palladium/C were added. The reaction mixture was placed under hydrogen atmosphere and stirred for 1 day. Palladium/C was filtered and the solvent was removed in vacuo. The oily product was dissolved in EtOAc, and ether saturated with gaseous HCl was added. The corresponding hydrochloride product was filtered and recrystallized in methanol. Yield 78%; mp >240 °C; ^{1}H NMR (300 MHz, DMSO-d_6), δ = 3.00 (m, 4H, CH_2), 3.30 (s, 3H, NCH_3), 7.11 (dd, 1H, H_5, J = 7.9 Hz, J_{5-7} = 1.2 Hz), 7.21 (d, 1H, H_4, J = 7.9 Hz), 7.30 (d, 1H, H_7, J = 1.2 Hz), 8.10 (br s, 3H, NH_3^+); Mass *m/z* 193.3 (M^++1).

4.4. General procedure for the synthesis of benzamide derivatives (9a–c)

To a solution of 10% K_2CO_3 aqueous (20 mL) and chloroform (30 mL), 6-(2-ethylamino)-3-methyl-2(*3H*)-benzothiazolone or benzoxazolone (**5a** or **5b**) (10 mmol) and the desired phenyl acid chloride (15 mmol) were added. The reaction mixture was stirred for 2 h at room temperature. A 5% NaOH aqueous solution was added (10 mL) and the reaction mixture was stirred for 30 min. The organic layer was dried with magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was recrystallized in the appropriate solvent.

4.4.1. 3-Methoxy-*N*-[2-(3-methyl-2-oxo-2,3-dihydro-benzothiazol-6-yl)ethyl]benzamide (9a)

Yield 78% (absolute ethanol); mp 130–131 °C; ^{1}H NMR (300 MHz, $CDCl_3$), δ = 2.95 (t, 2H, CH_2, J = 7.18 Hz), 3.40 (s, 3H, NCH_3), 3.70 (t, 2H, CH_2, J = 7.28 Hz), 3.85 (s, 3H, OCH_3), 6.35 (s, 1H, NH), 6.97 (d, 1H, H_4, J = 8.2 Hz), 7.03 (dd, 1H, H_5, J_{5-4} = 8.02 Hz, J_{5-7} = 1.17 Hz), 7.18 (d, 1H, H_{Ar}, J = 7.24 Hz), 7.23 (d, 1H, H_7, J = 1.17 Hz), 7.30 (m, 3H, H_{Ar}).; Mass *m/z* 343.4 (M^++1).

4.4.2. 3-Methoxy-*N*-[2-(3-methyl-2-oxo-2,3-dihydro-benzoxazol-6-yl)ethyl]benzamide (9b)

Yield 69% (absolute ethanol); mp 137–138 °C; ^{1}H NMR (300 MHz, $CDCl_3$), δ = 2.95 (t, 2H, CH_2, J = 6.5 Hz), 3.40 (s, 3H, NCH_3), 3.70 (t, 2H, CH_2, J = 6.5 Hz), 3.85 (s, 3H, OCH_3), 6.35 (s, 1H, NH), 6.97 (d, 1H, H_4, J = 8.2 Hz), 7.03 (dd, 1H, H_5, J = 8.2 Hz J = 1.2 Hz), 7.18 (d, 1H, H_{Ar}, J = 7.3 Hz), 7.23 (d, 1H, H_7, J = 1.2 Hz), 7.30 (m, 3H, H_{Ar}); Mass *m/z* 327.5 (M^++1).

4.4.3. 3-Benzyloxy-*N*-[2-(3-methyl-2-oxo-2,3-dihydro-benzoxazol-6-yl)ethyl]benzamide (9c)

Yield 94% (acetonitrile); mp 178–179 °C; ^{1}H NMR (300 MHz, $CDCl_3$), δ = 2.96 (t, 2H, CH_2, J = 6.4 Hz), 3.42 (s, 3H, NCH_3), 3.70 (t, 2H, CH_2, J = 6.4 Hz), 5.10 (s, 2H, OCH_2), 6.20 (s, 1H, NH), 6.90 (d, 1H, H_4, J = 7.9 Hz), 7.03 (dd, 1H, H_5, J = 7.9 Hz, J = 1.7 Hz), 7.10 (m, 4H, H_{Ar}), 7.37 (m, 1H, H_7), 7.40 (m, 5H, H_{Ar}); Mass *m/z* 403.3 (M^++1).

4.5. General procedure for the synthesis of derivatives 10a–c

To a solution of acetonitrile (50 mL), benzamide derivatives (**9a–c**) (32 mmol) and $POCl_3$ (3.1 mL, 320 mmol) were added. The reaction mixture was refluxed for 1 day and then evaporated under reduced pressure. The crude residue was washed with ethyl acetate and filtered. The precipitate was recrystallized in the appropriate solvent.

4.5.1. 5-(3-Methoxyphenyl)-3-methyl-7,8-dihydro-*3H*-1-thia-3,6-diaza-benz[*f*]inden-2-one hydrochloride (10a)

Yield 68% (ethanol 95°); mp >240 °C; ^{1}H NMR (300 MHz, DMSO-d_6), δ = 3.20 (t, 2H, CH_2, J = 6.4 Hz), 3.35 (s, 3H, NCH_3), 3.90 (s, 3H, OCH_3), 4.00 (t, 2H, CH_2, J = 6.4 Hz), 7.20 (s, 1H, H_4), 7.35 (m, 2H, H_{Ar}), 7.50 (d, 1H, H_{Ar} J = 1.8 Hz), 7.60 (t, 1H, H_{Ar}, J = 7.8 Hz), 8.00 (s, 1H, H_9) 9.50 (br s, 1H, NH^+); Mass *m/z* 325.2 (M^++1).

4.5.2. 5-(3-Methoxyphenyl)-3-methyl-7,8-dihydro-*3H*-1-oxa-3,6-diaza-benz[*f*]inden-2-one hydrochloride (10b)

Yield 66% (ethanol 95°); mp >240 °C; ^{1}H NMR (300 MHz, DMSO-d_6), δ = 3.20 (t, 2H, CH_2, J = 7.3 Hz), 3.30 (s, 3H, NCH_3), 3.85 (s, 3H, OCH_3), 4.00 (t, 2H, CH_2, J = 7.3 Hz), 7.30 (m, 2H, H_4, H_{Ar}), 7.40 (m, 2H, H_{Ar}), 7.60 (t, 1H, H_{Ar}, J = 7.5 Hz), 7.70 (s, 1H, H_9), 10.20 (s, 1H, NH^+); Mass m/z 309.5 (M^++1).

4.5.3. 5-(3-Benzyloxyphenyl)-3-methyl-7,8-dihydro-*3H*-1-oxa-3,6-diaza-benz[*f*]inden-2-one hydrochloride (10c)

Yield 45% (acetonitrile); mp >240 °C; ^{1}H NMR (300 MHz, DMSO-d_6), δ = 3.20 (t, 2H, CH_2, J = 7.4 Hz), 3.30 (s, 3H, NCH_3), 4.00 (t, 2H, CH_2, J = 7.4 Hz), 5.20 (s, 2H, OCH_2), 7.25 (s, 1H, H_4), 7.40 (m, 8H, H_{Ar}), 7.60 (s, 1H, H_9), 10.20 (br s, 1H, NH^+); Mass m/z 385.6 (M^++1).

4.6. General procedure for the synthesis of derivatives 11a–c

In 50 mL of methanol, derivatives **10a**–**c** (24 mmol) were solubilized and then sodium borohydride (60 mg, 36 mmol) was added portionwise. The mixture was added for 1 h at room temperature and then evaporated under reduced pressure. Water (30 mL) and chloroform were added to the residue. The organic layer was dried with magnesium sulfate, filtered, and concentrated under reduced pressure. The oily product was solubilized in dry ethyl acetate and treated with gaseous hydrochloric acid in diethyl ether (20 mL). The precipitate was filtered and washed with ethyl acetate. The residue was recrystallized with the appropriate solvent.

4.6.1. 5-(3-Methoxyphenyl)-3-methyl-5,6,7,8-tetrahydro-*3H*-1-thia-3,6-diaza-benz[*f*]inden-2-one hydrochloride (11a)

Yield 83% (ethanol 95°); mp >240 °C; ^{1}H NMR (300 MHz, DMSO-d_6), δ = 3.20–3.45 (m, 7H, CH_2, CH_2, NCH_3), 3.75 (s, 3H, OCH_3), 5.80 (s, 1H, CH), 6.70 (s, 1H, H_4), 6.85 (d, 1H, H_{Ar}, J = 7.8 Hz), 7.00 (dd, 1H, H_{Ar}, J = 8.2 Hz, J = 2.0 Hz), 7.10 (t, 1H, H_{Ar}, J = 1.8 Hz, J = 2.0 Hz), 7.30 (t, 1H, H_{Ar}, J = 8.2 Hz, J = 7.8 Hz), 7.60 (s, 1H, H_9), 9.70 (br s, 1H, NH_2^+), 10.50 (br s, 1H, NH_2^+); Mass m/z 327.4 (M^++1).

4.6.2. 5-(3-Methoxyphenyl)-3-methyl-5,6,7,8-tetrahydro-*3H*-1-oxa-3,6-diaza-benz[*f*]inden-2-one hydrochloride (11b)

Yield 74% (ethanol 95°); mp >240 °C; ^{1}H NMR (300 MHz, DMSO-d_6), δ = 3.10 (m, 7H, CH_2, CH_2, NCH_3), 3.75 (s, 3H, OCH_3), 5.75 (s, 1H, CH), 6.60 (s, 1H, H_4), 6.80 (d, 1H, H_{Ar}, J = 7.6 Hz), 7.05 (dd, 1H, H_{Ar}, J = 7.6 Hz, J = 1.1 Hz), 7.10 (s, 1H, H_{Ar}), 7.30 (s, 1H, H_9), 7.40 (t, 1H, H_{Ar}, J = 7.6 Hz, J = 7.9 Hz), 9.70 (br s, 1H, NH_2^+), 10.50 (br s, 1H, NH_2^+); Mass m/z 311.5 (M^++1).

4.6.3. 5-(3-Benzyloxyphenyl)-3-methyl-5,6,7,8-tetrahydro-*3H*-1-oxa-3,6-diaza-benz[*f*]inden-2-one hydrochloride (11c)

Yield 91% (acetonitrile); mp >240 °C; ^{1}H NMR (300 MHz, DMSO-d_6), δ = 3.30 (m, 7H, CH_2, CH_2, NCH_3), 5.10 (s, 2H, OCH_2), 5.75 (s, 1H, CH), 6.55 (s, 1H, H_4), 6.90 (d, 1H, H_{Ar}, J = 7.8 Hz), 7.05 (t, 1H, H_{Ar}, J = 1.5 Hz), 7.10 (dd, 1H, H_{Ar}, J = 7.7 Hz, J = 1.9 Hz), 7.40 (m, 7H, H_9, H_{Ar}), 9.70 (br s, 1H, NH_2^+), 10.50 (br s, 1H, NH_2^+); Mass m/z 387.3 (M^++1).

4.7. General procedure for the synthesis of derivatives 12a–d

Propyl or nonyl iodide (10 mmol) was added to a solution of compounds **8a**–**c** (10 mmol) and potassium carbonate (4.2 g, 30 mmol) in CH_3CN. The reaction mixture was refluxed for 1 day and then evaporated. Ethyl acetate (80 mL) and water (50 mL) were added. The organic layer was dried with magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography using dichloromethane/methanol (98/2, v/v) as eluent to afford compounds **12a**–**d**. The oily product was solubilized in dry ethyl acetate and treated with gaseous hydrochloric acid in diethyl ether (20 mL). The precipitate was filtered and washed with ethyl acetate. The residue was recrystallized with the appropriate solvent.

4.7.1. 5-(3-Methoxyphenyl)-3-methyl-6-propyl-5,6,7,8-tetrahydro-3*H*-1-thia-3,6-diaza-benz[*f*] inden-2-one hydrochloride (12a)

Yield 64% (absolute ethanol); mp >240 °C; ^{1}H NMR (300 MHz, DMSO-d_6), δ = 0.80 (t, 3H, CH_3, J = 7.3 Hz), 1.50 (m, 2H, CH_2), 2.30 (m, 2H, CH_2), 2.50 (m, 2H, CH_2) 3.10 (m, 5H, CH_2, NCH_3), 3.80 (s, 3H, OCH_3,), 4.60 (s, 1H, CH), 6.40 (s, 1H, H_4), 6.80 (m, 4H, H_{Ar}), 7.15 (s, 1H, H_9), 11.60 (br s, 1H, NH^+); Mass m/z 369.4 (M^++1).

4.7.2. 5-(3-Methoxyphenyl)-3-methyl-6-propyl-5,6,7,8-tetrahydro-3*H*-1-oxa-3,6-diaza-benz[*f*]inden-2-one hydrochloride (12b)

Yield 68% (absolute ethanol); mp >240 °C; ^{1}H NMR (300 MHz, DMSO-d_6), δ = 0.80 (t, 3H, CH_3, J = 7.3 Hz), 1.80 (m, 2H, CH_2), 3.10 (m, 7H, CH_2 CH_2, NCH_3,), 3.60 (m, 5H, CH_2, OCH_3), 5.80 (s, 1H, CH), 6.50 (s, 1H, H_4), 6.90 (m, 3H, H_{Ar}), 7.20 (s, 1H, H_9), 7.40 (m, 1H, H_{Ar}), 11.50 (br s, 1H, NH^+); Mass m/z 353.5 (M^++1).

4.7.3. 5-(3-Benzyloxyphenyl)-3-methyl-6-propyl-5,6,7,8-tetrahydro-3*H*-1-oxa-3,6-diaza-benz[*f*]inden-2-one hydrochloride (12c)

Yield 76% (absolute ethanol); mp 158–159 °C; ^{1}H NMR (300 MHz, DMSO-d_6), δ = 0.80 (t, 3H, CH_3, J = 7.2 Hz), 1.80 (m, 2H, CH_2), 3.30 (m, 9H, CH_2, CH_2, CH_2, NCH_3), 5.10 (s, 2H, OCH_2), 5.80 (s, 1H, CH), 6.30 (s, 1H, H_4), 7.15 (m, 9H, H_{Ar}), 7.20 (s, 1H, H_9), 11.50 (br s, 1H, NH^+); Mass m/z 429.6 (M^++1).

4.7.4. 5-(3-Methoxyphenyl)-3-methyl-6-nonyl-5,6,7,8-tetrahydro-3*H*-1-oxa-3,6-diaza-benz[*f*]inden-2-one hydrochloride (12d)

Yield 63% (acetonitrile); mp 142–143 °C; ^{1}H NMR (300 MHz, DMSO-d_6), δ = 0.80 (t, 3H, CH_3, J = 7.1 Hz), 1,10–1,40 (m, 12H, CH_2), 1.50 (m, 2H, CH_2), 2.50 (m, 2H, CH_2), 2.70 (m, 2H, CH_2), 3.20 (m, 2H, CH_2), 3.70 (s, 3H, NCH_3), 3.70 (s, 3H, OCH_3), 5.40 (s, 1H, CH), 6.50 (s, 1H, H_4), 6.80 (d, 1H, H_{Ar}, J = 7.8 Hz), 7.00 (m, 2H, H_{Ar}), 7.20 (s, 1H, H_9), 7.35 (m, 1H, H_{Ar}), 11.10 (br s, 1H, NH^+); Mass m/z 437.6 (M^++1).

4.7.5. 5-(3-Hydroxyphenyl)-3-methyl-6-propyl-5,6,7,8-tetrahydro-3*H*-1-thia-3,6-diaza-benz[*f*]inden-2-one hydrochloride(13)

To a solution of compound **12c** (10 mmol) in methanol was added 10% Pd/C (100 mg). The resulting solution was stirred at room temperature under atmospheric pressure of hydrogen for 1 day. The solution was concentrated to dryness. The residue was purified by column chromatography using dichloromethane/methanol (90/10, v/v) as eluent to afford compound **13**. The oily product was solubilized in dry ethyl acetate and treated with gaseous hydrochloric acid in diethyl ether (20 mL). The precipitate was filtered and washed with ethyl acetate. The residue was recrystallized in acetonitrile. Yield 70%; mp 230–231 °C; ^{1}H NMR (300 MHz, $CDCl_3$), δ = 0.80 (t, 3H, CH_3, J = 7.3 Hz), 1.50 (m, 2H, CH_2), 2.30 (m, 1H, CH_2), 2.45 (m, 1H, CH_2), 2.60 (m, 1H, CH_2), 2.90 (m, 1H, CH_2), 3.05 (m, 1H, CH_2), 3.15 (m, 1H, CH_2), 3.20 (m, 3H, NCH_3), 4.55 (s, 1H, CH), 6.40 (s, 1H, H_4), 6.70 (m, 2H, H_{Ar}), 6.80 (d, 1H, H_{Ar}, J = 7.5 Hz), 6.95 (s, 1H, H_9), 7.20 (t, 1H, H_{Ar}, J = 7.8 Hz), 11.00 (br s, 1H, NH^+); Mass m/z 339.5 (M^++1).

4.8. General procedure for the synthesis of derivatives 14b and 14c

To a solution of compound **12b** or **13** (10 mmol) in methanol/water (20/10) was added sodium hydroxide (1.6 g, 40 mmol). The reaction mixture was refluxed for 1 h and then evaporated. Water (50 mL) was added. The aqueous solution was extracted with diethyl ether, acidified (pH 3) with 6 M HCl solution, and then

the pH was adjusted to 8 with aqueous solution of sodium bicarbonate. The precipitate was extracted with ethyl acetate. The organic layer was dried with magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography using dichloromethane/methanol (90/10, v/v) as eluent to afford compounds **14b–c**. The oily product **14b** was solubilized in dry ethyl acetate and treated with gaseous hydrochloric acid in diethyl ether (20 mL). The precipitate was filtered and washed with ethyl acetate.

4.8.1. 6-Hydroxy-1-(3-methoxyphenyl)-7-methylamino-2-propyl-1,2,3,4-tetrahydro-isoquinoline hydrochloride (14b)

Yield 58% (acetonitrile); mp >240 °C; ^{1}H NMR (300 MHz, DMSO-d_6), δ = 0.80 (t, 3H, CH_3, J = 7.1 Hz), 1.80 (m, 2H, CH_2), 2.70 (s, 3H, NCH_3), 3.00 (m, 2H, CH_2), 3.40 (m, 2H, CH_2), 3.70 (m, 2H, CH_2), 3.85 (s, 3H, OCH_3), 5.60 (s, 1H, CH), 6.60 (s, 1H, H_8), 7.00 (m, 4H, H_5, H_{Ar}), 7.30 (s, 1H, OH), 7.40 (m, 1H, H_{Ar}), 11.30 (br s, 2H, NH, NH^+); Mass m/z 327.3 (M^++1).

4.8.2. 6-Hydroxy-1-(3-hydroxyphenyl)-7-methylamino-2-propyl-1,2,3,4-tetrahydro-isoquinoline (14c)

Yield 68%; mp 140–141 °C; ^{1}H NMR (300 MHz, DMSO-d_6), δ = 0.80 (t, 3H, CH_3, J = 7.2 Hz), 1.60 (m, 2H, CH_2), 2.70 (m, 9H, CH_2, CH_2, CH_2, NCH_3), 4.60 (s, 1H, CH), 6.00 (s, 1H, H_8), 6.20 (s, 1H, H_{Ar}), 6.50 (s, 1H, H_5), 6.70 (dd, 1H, H_{Ar}, J = 7.9 Hz, J = 1.7 Hz), 6.80 (d, 1H, H_{Ar}, J = 7.4 Hz), 7.10 (t, 1H, H_{Ar}, J = 7.9 Hz, J = 7.4 Hz); Mass m/z 313.3 (M^++1).

5. Antioxidant activity

5.1. Low density lipoprotein (LDL) oxidation inhibition test

Human LDLs were isolated from freshly drawn blood from healthy, normolipidemic, fasting volunteers. LDLs were isolated by sequential density gradient ultracentrifugation in sodium bromide density solutions in the density range of 1.019–1.063 g/mL as previously reported.[37] The protein concentration was determined by Peterson's method[38] using bovine serum albumin as the standard. Oxidation of LDL was initiated by copper.

LDLs were extensively dialyzed against 0.01 M phosphate-buffered-saline (PBS), pH 7.4, under N_2 at 4 °C. Then oxidation was induced at 30 °C by adding 20 μL of 16.6 μM $CuSO_4$ or 2 mM AAPH to 160 μL of LDL (125 μg of protein/mL) in the presence or absence of 20 μl derivatives from 1 μM to 10 μM or PBS for control. During copper or AAPH-induced LDL oxidation, diene-conjugated formation was followed by the measurement of optical density at 234 nm every 10 min for 8 h at 30 °C with a thermostated Spectra Max Plus Molecular Devices spectrophotometer (96 wells, Molecular Devices Corporation Sunnyvale, California 94089).[39] Drug activities were expressed in R, the ratio between the length of the lag phase of the oxidative procedure of LDL in the presence of different compounds and the length of the one without compound (control). As their antioxidant capacity can be characterized by the evolution of the LDL oxidation curves along with their concentration, each compound was tested at concentrations of 1–100 μM. Air-oxidized LDLs (mO-LDL) are obtained by incubation of LDL at 30 °C without $CuSO_4$ or AAPH.

5.2. Cellular cytotoxicity

Bovine aortic endothelial cells (BAECs) were isolated as described by Gospodarowicz[40] and cultured in Dubelco's-modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum, 600 ng/mL glutamine, and 100 U/mL penicillin. BAECs were used under passage 10. BAECs were then subcultured in 6-well plates. 10 μL drugs in methanol solution (1 μM) were added to the culture medium of cells for 24 h. Then cells were incubated at 37 °C during a period of 16 h in the presence of LDL or minimally oxidized LDL (mO-LDL) at 100 μg/mL with an addition of 10 μL methanol or drugs in methanol solution (final concentration 10 μM).[41] After 16 h of incubation, Lactate Dehydrogenase (LDH) activity in the medium was measured by the spectrometric analysis of NAD reduction using the LDH kit (sigma, Saint Quentin Fallavier, France). LDH activity was expressed as the percentage of total LDH cellular release following the addition of Triton X-100 (final concentration of 0.1% v/v in ethanol). The results are the mean (±SD) of two different incubations each performed in triplicate.

Cellular vitality was determined by measuring mitochondrial activity in the presence of MTT:(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). MTT is reduced to purple formazan in the mitochondria of living cells. 100 μL of a 5 mg solution of MTT in PBS were added to each culture well and incubated for 5 h. Following this period, the medium was removed and the crystals were dissolved in 4 mL of dimethylformamide. Absorbance was read at 590 nm, and the results were expressed as the percentage of the value of control wells.[42]

5.3. Data analysis

The results were expressed as mean ± SD when a minimal number of three independent experiments were performed in triplicate. Differences between the experimental results were tested using the Mann Whitney test.

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