Bioorganic & Medicinal Chemistry 22 (2014) 4269–4276

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis, antioxidant and cytoprotective evaluation of potential antiatherogenic phenolic hydrazones. A structure–activity relationship insight

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ARTICLE INFO

Article history:
Received 25 March 2014
Revised 12 May 2014
Accepted 14 May 2014
Available online 23 May 2014

Keywords:
Phenolic antioxidants
Arylhydrazones
Oxidative stress
LDL oxidation
Atherosclerosis

ABSTRACT

A novel series of hydrazones derived from substituted benzaldehydes have been synthesized as potential antiatherogenic agents. Several methods were used for exploring their antioxidant and cytoprotective properties, such as their scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, the inhibition of superoxide anion ($O_2^{\cdot-}$) generation and the measurement of cell-induced low-density lipoprotein oxidation (monitored by the formation of TBARS). The cytoprotective efficacy was also evaluated by measuring the cell viability (monitored by the MTT assay) in the presence of cytotoxic oxidized LDL. In this report, we discuss the relationship between the chemical structure of phenolic hydrazones and their antioxidant and cytoprotective activities, for subsequent application as antiatherogenic agents. This SAR study confirms that the phenolic frame is not the only prerequisite for antioxidant activity and *N*-methylbenzothiazole hydrazone moiety magnifies the dual required properties in two most interesting derivatives.

1. Introduction

Atherosclerosis is a complex multifactorial disease characterized by the modification and oxidation of low density lipoproteins (LDL) that, together with a chronic inflammatory response in the artery wall, leads to atherosclerotic lesions.[1] To date, considerable evidence supports a role for oxidatively modified LDL (oxLDL) related to oxidative stress in the pathogenesis of atherosclerosis.[2] Reactive oxygen species (ROS) are produced in cellular metabolism through different pathways, but in healthy individuals, they are rapidly eliminated by a wide range of antioxidant systems designed to prevent their harmful effects. When the prooxidant/antioxidant balance is perturbed, due to either an abnormal production of ROS or depletion of antioxidant defenses, a situation called oxidative stress arises.[3] Continued oxidative stress leads to cellular damage, due to alteration of lipids, enzymes, proteins, and DNA. An abnormal production of superoxide anion ($O_2^{\cdot-}$) by the endothelium occurs in the atherosclerotic vascular changes,[4] which in turn contributes to enhance the oxidation of low density lipoproteins (LDL) in the subendothelial space.[5] This is the first step in a complex process that leads first to the formation of foam cells, then of the fatty streak, and ultimately to atherosclerotic plaque.[5]

Polyphenols are known to be powerful antioxidants and their antioxidative capacity is related to their chemical structure. They have redox properties allowing them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers that contribute to their antioxidant capacity.[6] Due to the central role of oxidative stress in the pathogenesis of atherosclerosis and the inhibition of atherosclerosis progression by antioxidants supplementation in animal models,[7] natural or non-natural phenolic antioxidant agents may be of therapeutic benefit for atherosclerosis.

Hydrazones scaffolds exhibit various biological properties,[8] in particular for their ability to upregulate LDL receptor to treat hypercholesterolemia.[9]

In our continuing effort to develop new antiatherogenic agents, we have recently reported the antioxidant activities of a series of phenolic hydrazones derived from syringaldehyde.[10] These first results and the inadequate single-targeted therapeutic approach in multifactorial disorders such as atherosclerosis,[11] prompted us to extend this study toward an evaluation of the structure activity

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http://dx.doi.org/10.1016/j.bmc.2014.05.034
0968-0896/

relationship, for designing new dual agents with antioxidant and cytoprotective properties.

The objectives of the present study were to assess whether some of these phenolic hydrazones could prevent oxidative stress. We investigated the physicochemical properties on 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH) and radical superoxide anion ($O_2^{\cdot-}$) scavenging activities. These series of compounds were further used to study their protective effect against LDL oxidation induced by murine endothelial cells and their cytoprotective effect against oxLDL toxicity. This study helps us to explore structure–activity-relationships in terms of the antioxidant and cytoprotective properties of these compounds.

2. Results and discussion

2.1. Chemistry

A series of 33 hydrazones derivatives were efficiently synthesized by condensation of various hydrazines (1-hydrazinophtalazine hydrochloride, isonicotinyl acid hydrazide, benzyloxycarbonylhydrazine, *N*-benzyloxycarbonyl-2-methylhydrazine hydrochloride,[12] 2-hydrazinobenzothiazole, 2-(1-methylhydrazinyl)benzothiazole,[13] *N*-aminorhodanine) and commercially available benzaldehydes in refluxing ethanol. In regards to our previously reported results on syringic (3,5-dimethoxy-4-hydroxyphenyl moiety) hydrazones **1**, we chose to modulate the aromatic part substitution by preparing hydrazones **2** starting from 3,4,5-trimethoxybenzaldehyde (non phenolic ones) or phenolic ones **3**–**7** starting respectively from 3, 4-hydroxy-5-methoxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde, 3,4-dihydroxybenzaldehyde, 2-hydroxybenzaldehyde, 2, 4-hydroxy-5-methoxybenzaldehyde (Table 1).

2.2. Antioxidant activities

Free radicals have been suggested to be important mediating agents in aging and several human diseases, including cardiovascular diseases.[14] During the past decade, discovery of new antioxidants, of both synthetic and natural origin, has been a major focus in many laboratories. Many natural and synthetic phenol derivatives have been reported to have protective effects in pathological processes caused by oxidative stress. In the first part of the present study we assessed the capacity of a series of phenolic hydrazones to reduce free radicals by using a model reaction with stable free DPPH radical and a qualitative non-enzymatic radical superoxide anion scavenging assay.

2.2.1. DPPH radical scavenging activity

The in vitro antioxidant profile of the synthesized hydrazones was determined by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay,[15] which measures the hydrogen-donating ability of antioxidants to convert stable DPPH free radical to 2,2-diphenyl-1-picrylhydrazine. The reaction is accompanied by a change in color from deep-purple to light-yellow and is monitored spectrophotometrically at 517 nm. All tested hydrazones present UV absorbance between 230 and 350 nm and do not interfere with the kinetic measurement of DPPH absorbance decrease at 517 nm, except for hydrazones **3a**, **1e**, **3e**, **4e** and **1f** which form a colored species in the presence of DPPH, preventing the measurement. Trolox was used as positive reference antioxidant[16] and results are disclosed in Table 1.

The non-phenolic hydrazones **2b**, **2c**, **2d** and **2f** (Table 1, entries 7, 14, 21 and 29) do not present any radical scavenging activity as expected. However non-phenolic hydrazones **2a** and **2e** (Table 1, entries 2 and 24) keep a moderate DPPH trapping capacity ($IC_{50} \sim 40$ μM) which could be attributed to the NH hydrazones participation of the latter compounds to the antiradical capacity.

The catechol derivatives **5a**, **5b**, **5c** and **5e** (Table 1, entries 5, 10, 17 and 27) exhibit the best radical scavenging activity (6.1 μM < IC_{50} < 7.3 μM). In the presence of an added 5-methoxy group on the catechol counterpart (**3b** and **3c**), the antiradical activity decreases but remains in the range of Trolox antiradical capacity ($IC_{50} \sim 20$ μM) (Table 1, entries 8 and 15). The trisubstituted 2, 4-hydroxy-5-methoxyphenyl hydrazones **7b** and **7c** (Table 1, entries 12 and 19) possess a slightly better DPPH scavenging activity than the previous ones. The second hydroxy group contributes to the antiradical activity even if it is in position 2 or 3 on the phenolic moiety.

2.2.2. Radical superoxide anion scavenging activity

Oxidative stress generates reactive oxygen species, including superoxide anion and nitric oxide radical ($NO^{\cdot}$) and contributes to the endothelium dysfunction in atherosclerosis, either by reacting with NO, resulting in peroxynitrite formation[17] or by stimulating the conversion of LDL to oxLDL. We have evaluated the $O_2^{\cdot-}$ scavenging effect by using the non enzymatic system phenazine methosulfate-nicotinamide adenine dinucleotide-nitroblue tetrazolium (PMS-NADH-NBT).[18] PMS oxidizes NADH and subsequently reduces dissolved oxygen to form $O_2^{\cdot-}$ which in turn, reduces NBT to a colored product (a blue formazan). The latter was quantified spectrophotometrically at 560 nm. Superoxide-scavenging antioxidants detoxify superoxide decreasing the extent of NBT reduction can therefore be expressed as the percent inhibition of NBT reduction.

The hydrazones families **a**, **c**, **d**, **e** and **f** were evaluated at 10 μM concentration as for the antioxidant and cytoprotective assays (see below) and compared with chlorogenic acid and trolox[19] (Figure 1). All the tested hydrazones reveal a moderate $O_2^{\cdot-}$ scavenging effect except derivatives **3a**, **3c**, **1e**, **2e**, **3e** and **1f** which show comparable or better activity (36.7 ± 3.1%, 41.4 ± 5.9%, 35.0 ± 3.2, 34 ± 2.1, 41.7 ± 5.2% and 43.3 ± 4.6%, respectively) than chlorogenic acid (35.5 ± 1.1%) and trolox (22.2 ± 1.1%). These results highlight that 4-hydroxy-3,5-dimethoxyphenyl and the methoxycatechol scaffolds play an important role to scavenge superoxide anion independently of the chemical structure of the hydrazones frame.

2.3. Protective activities against LDL oxidation (TBARS) and oxLDL toxicity (MTT)

We aimed to determine the antioxidant ability of the newly synthesized aryl-hydrazones in inhibiting LDL oxidation mediated by cell contact with murine aortic endothelial cells (CRL2181-ATCC), this system allowing to mimic the pathophysiological events occurring in the vascular wall.[20]

LDL oxidation was determined by using the thiobarbituric acid reactive substance (TBARS) assay and expressed as a percentage of TBARS formed in the experiment with native LDL in contact with CRL2181 endothelial cells in the absence of antioxidant.[21] Cytoprotection was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. All agents were initially tested at 10 μM which we previously established as the optimum concentration to minimize the self-toxicity,[22] and at 1 μM as the discriminating concentration. The results are reported in Table 1.

Most of the prepared hydrazones exhibit better abilities than trolox and α-tocopherol. In the hydralazones family (Table 1, entries 1–5), all agents present an excellent antioxidant efficiency (3–4%) and cytoprotective activity (66–100%) against oxLDL toxicity at 10 μM except for the trimethoxyl derivative **2a**. It is noteworthy that the latter shows obvious toxicity even at 1 μM as all the derivatives **2** possessing a trimethoxyaryl scaffold (Table 1, entries 2, 7, 14, 24 and 29). This fragment carrying toxicity becomes thus unattractive for the dual biological desired properties. At 1 μM, only compound **3a** (Table 1, entry 3) possessing a methoxy-catechol-phenyl frame remains interesting but weaker antioxidant