ASSESSMENT OF ANTIOXIDANT CAPACITY *IN VITRO* OF SYNTHETIC COMPOUNDS CONTAINING A 4-(SUBSTITUTED PHENYL)-/4-(DIPHENYLMETHYL)PIPERAZIN-1-YL STRUCTURAL MOTIF, PROMISING CARDIOVASCULAR DRUGS, OR ANTIMYCOBACTERIAL AGENTS

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ABSTRACT

Biological significance of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) is rather complex and paradoxical. Elevated levels of ROS have commonly been implicated in a variety of pathological conditions and diseases, including cardiovascular diseases, cancer, inflammation, and neurodegenerative diseases as well. On the other hand, ROS and RNS are generated immediately after the recognition of any invading foreign pathogens such as viruses, or bacteria to destroy them. *Mycobacterium tuberculosis* is a causative agent of human tuberculosis, an ancient disease, which is one of the major leading causes of infectious deaths globally. During a phagocytosis process, *M. tuberculosis* evades the host defense mechanisms to prolong their existence in humans. Activation of pro-inflammatory cytokines by ROS/RNS in macrophages during phagocytosis is important in eliminating the pathogen by host immunity. However, the *mycobacterium* has found ways to downregulate the inflammatory response and ROS/RNS produced in macrophages. In current research, a series of hybrid compounds, promising cardiovascular/antimycobacterial agents, which contain a phenylcarbamoyloxy moiety, connecting 2-hydroxypropan-1,3-diyl chain and 4-(substituted phenyl)-/4-(diphenylmethyl)piperazin-1-yl group was *in vitro* evaluated in order to inspect their potential to reduce a DPPH radical. Found moderate antioxidant capacity of 1-[2-hydroxypropyl-3-(phenylcarbamoyloxy)]-4-(4-methoxyphenyl)piperazin-1-ium chloride (**6g**)

might be the favorable feature if focusing on this molecule as an effective cardiovascular drug. On the other hand, 1-[2-hydroxypropyl-3-(phenylcarbamoyloxy)]-(3,4-dichlorophenyl)piperazin-1-ium chloride (**6b**) might supposed to be an effective anti-TB agent in terms of its very suitable structural arrangement and selection of substituents within a basic fragment. Among currently screened derivatives, the compound **6b** showed the lowest potential to reduce the radical, thus, this property would not weaken its assumed anti-TB activity.

None of assessed synthetic compounds have proved antioxidant capacity of reference Trolox molecule.

Keywords: ROS, RNS, cardiovascular drugs, antimycobacterial agents, antioxidant capacity, piperazin compounds with aromatic rings.

INTRODUCTION

Oxidative stress is defined as a disbalance caused by aggregation and production of ROS [1] and RNS. Although the understanding of the role of ROS and RNS in human cellular functioning has crucially changed and adapted over past years, their functions are still not fully revealed and they are, as well as the generation of ROS/RNS, crucial when approaching oxidative stress. In the first part of this research work, the emphasis is going to be on how and where ROS/RNS are formed and detoxificated, how they contribute to body physiological functions and their linkage to pathologies.

Although oxygen is crucial and inalienable for human life and basic aerobic metabolic processes, under certain situations it could evolve into potential harmful chemical compounds, namely Reactive Oxygen Species (ROS) [2].

ROS are a group of reactive molecules derived from molecular oxygen having the potential to cause a molecular damage leading to a number of deleterious events. RNS are a group of nitrogen moieties associated with oxygen, having the same damaging potential as ROS. They are formed when nitric oxide (NO) produced either exogenously or endogenously interacts with ROS like superoxide ($O_2^{\bullet -}$), and hydrogen peroxide (H_2O_2). ROS/RNS may or may not be radicals in nature. Mechanism through they cause the damage (not only) in human body are free-radical reactions. Examples of ROS include superoxide anion ($O_2^{\bullet -}$), peroxide ($O_2^{\bullet 2-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\bullet$), peroxyl radical ($RO_2\bullet$), singlet oxygen ($^1O_2$), ozone (O_3). Examples of RNS include nitric oxide ($NO\bullet$), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl).

ROS are considered to be byproducts during the mitochondrial electron transport of aerobic respiration (mitochondrial oxidative metabolism) as well as, together with RNS, they are

formed during another aerobic metabolism and in cellular response to xenobiotics, cytokines, and bacterial and viral invasion. As regards their beneficial effects, initially it was supposed that ROS/RNS are generated only by phagocytic cells as their part of defence mechanisms. Recent work has showed that ROS/RNS have also a role in cell signalling (ROS as both intra- and intercellular messengers), including apoptosis; gene expression; and the activation of cell signalling cascades.

Thus ROS/RNS are formed constantly in human body as products of normal cellular processes and have multiple cell locations of generation, but ROS mostly originate in mitochondria under pathological as well as physiological conditions.

Superoxide anion radical ($O_2^{\bullet -}$) during low pH environment, for instance, is the most abundant ROS and it is generated in the mitochondrial compartments by autooxidation reactions, enzymatic and non-enzymatic processes. Enzymes producing superoxide comprise lipoxygenase and xanthine oxidase [1]. But it is produced in mitochondria at several sites e.g. complex I, III, pyruvate dehydrogenase, glycerol 3-phosophate dehydrogenase and Q oxidoreductase [3]. Another ROS, hydrogen peroxide, is produced by NADPH oxidases in the mitochondria. The enzymes necessary for the conversion of oxygen to H_2O_2 comprise superoxide dismutase 1 and 2. Moreover, hydroxyl radicals (OH•) are established from H_2O_2 when iron, specifically ferrous ions are available [4]. Other sites of ROS production in mitochondria comprise NADH-cytochrome b5 reductase, succinate dehydrogenase and monoamine oxidase.

Peroxisomes are considered to be another source of ROS, generated as byproducts during catalytic action. H_2O_2, superoxide radical, hydroxyl radical and nitric oxide are given rise to by peroxisomes. $O_2^{\bullet -}$ formation is catalyzed by xanthine oxidase [5].

Through the endoplasmic reticulum (ER), ROS can additionally evolve. The microsomal monooxygenase system consists of several cytochrome P450 types, NADPH P450 reductase and cytochrome b5 and accelerates the reaction of exogenous compounds, thereby generating H_2O_2 and superoxide radicals. NADPH oxidases families, specifically NOX1 and NOX4 are also known to generate H_2O_2 through protein disulfide isomerase and are involved in liberating H_2O_2 into the endoplasmic reticular lumen.

Now I will focus on the enzymes themselves regardless of their place of action. A major group of ROS producing enzymes are oxidases. Xanthine oxidase produces ROS by metabolizing purine substances thereby oxidating hypoxanthine to xanthine and eventually to uric acid [6]. Additionally, xanthine oxidase is considered to be a potential generator of reactive oxygen species in vascular tissue [7]. Also, it catalyzes the reduction of nitrates to nitric oxide, whereas

nitric oxide can also be produced from L-arginine by nitric oxide synthase. Diamine oxidase participates in the oxidation of polyamines e.g. putrescine, spermidine and histamine, thereby generating H_2O_2. This enzyme is tissue specific and is found in kidneys, small intestine and liver. Cancer in these tissues show increased diamine oxidase activity. Cyclooxygenases (COX) and lipoxygenases (LOX) oxygenate arachidonic acid during polyunsaturated fatty acids metabolism, which goes along with prostaglandin G2 and H2 and hydroperoxide production. Hydroperoxide metabolism induces peroxyl and alkoxyl radicals. COX-1 and COX-2 as well as COX-3 possess different functions, but are all upregulated in precancerous and cancerous stages. Another enzyme which knowingly metabolizes neurotransmitters such as norepinephrine, serotonin and dopamine, is monoamine oxidase, which in elevated levels produces ROS. Other enzymes, for instance mitogen-activated kinases, cAMP-dependent protein kinases and PKB/AKT, can phosphorylate NOXs and increase its level and thus also ROS levels. Considering the genetic component which is able to generate ROS as well, it is important to contemplate p53.

However, also specific signaling pathways can result in the same outcome. Namely, NOXs, which are multimeric enzymes between the cellular membrane, can generate ROS upon stimulation and formation of functional NOX enzymes. Additionally, the PI3K/AKT/PTEN signaling pathway along with PKC activation accumulate NOX enzymes and hence enhance ROS production.

Since ROS generation in the body is inevitable, the neutralizing effect of the antioxidant system must be intact for maintaining proper balance between the ROS generation and antioxidant system. If there is an impaired removal of ROS and an extensive production, tissue becomes damaged and dysfunctional. Mitochondrial ROS production is balanced as its strong antioxidant system is present, however when radical production increases uncontrollably, mitochondrial damage might occur [8]. The mitochondria contains its own DNA and is known to be vulnerable to ROS and its damage leads to mutation and furthermore to dysfunction. Superoxide anions arise from mitochondrial DNA damage, which itself is a primary site of attack. This damage leads to oxidative stress and cellular and genomic instability. Additionally, proteins important for electron transport are being translated leading to increased ROS production, dysfunction of cell structures and finally apoptosis. Concordantly, crucial molecules including lipids, DNA as well as proteins are destroyed and accumulate, also favoring cell apoptosis [9].

Apart from general systematic inflammation that ROS and/or RNS cause, cardiovascular diseases also arise from oxidative stress. Endothelial damage is the pathological state of the

endothelium which can evolve into a cardiac disease. The reaction of superoxide and free radical NO forms $ONOO^-$, which in larger amounts injure because it is being converted to peroxynitrous acid thereby modifying protein structures. Reactive oxygen species are recognized to cause blood flow mismatch, arterial wall remodeling and shear stress [10].

In order to prevent or overcome the damages that are caused by reactive oxygen species, the body utilizes antioxidants. They function as radical scavenger, hydrogen donor, electron donor or enzyme inhibitor and are found in an enzyme or non-enzyme form.

The enzymatic forms act on the detoxification pathway by catalyzing the breakdown and decomposition of ROS. They include superoxide dismutase, which exists in three forms (SOD1, SOD2 and SOD3, all being in a particular locations within the cell) and accelerates the breakdown of superoxide anions to oxygen and hydrogen peroxide. Oxidase catalyzes the conversion of hydrogen peroxide to water and oxygen and is mostly found in the liver.

Non-enzymatic forms act as reducers or neutralizers and include exogenic and endogenic sources. Glutathione, with its thiol group in the cysteine part shows reducing activity, reduces metabolites and acts on oxidants as well. Vitamin C is one of the most commonly known exogenous substance with antioxidant properties. Due to its reaction with glutathione is kept in a reduced state thereby removing hydrogen peroxide. Vitamin E, also an exogenous source of antioxidant, can prevent lipid peroxidation chain reaction. Its reusable effect is maintained by its reduction through ascorbate or retinol. This mechanism is called redox recycling. An endogenous source includes melatonin (or N-acetyl-5-methoxytryptamine), which is produced by the pineal gland and has crucial antioxidant properties by protecting the membrane from lipid peroxidation.

Generally, there are different ways and levels of antioxidant action. The first line inhibits the overall formation of ROS, for instance, glutathione prevents lipid peroxidation. The second line scavenge the radicals to prevent further reactions, which include vitamin C, vitamin E or ubiquinol or thiols. The third line includes antioxidants like proteases or proteolytic enzymes within the cell that degrade oxidated substances or proteins by recognizing them early enough to prevent further initiations. The last line of defence is where the adaptation is sustained and antioxidants are mediated towards the site of radical production [11].

In experimental part of my work I focused on second line of antioxidant action and the main purpose was to preliminary investigate *in vitro* the antioxidant activity of a set of hybrid synthetic molecules containing a 4-(substituted phenyl)-/4-(diphenylmethyl) piperazin-1-yl structural motif, promising cardiovascular drugs, or antimycobacterial agents in connection with their possible clinical use.

Screened compounds with good antioxidant capacity might be beneficial for ROS and/or RNS-induced cardiovascular diseases while those with low antioxidant capacity might supposed to be an effective antimycobacterial agents as for drugs fighting against mycobacteria is assumed to be a beneficial not to have antioxidant activity not to destroy ROS and/or RNS as release of ROS/RNS by macrophages undergoing phagocytosis is crucial for the efficiency of the immune system [12] and exposing bacteria to ROS might be effective against bacterial infections [13].

MATERIALS

Synthetic compounds **6a**-**6e** and **6g** (samples) presently tested *in vitro* for their antioxidant capacity were synthesized by PharmDr. Mária Pecháčová under the supervision of Assoc. Prof. PharmDr. Ivan Malík, PhD. at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Comenius University in Bratislava.

Chemical names and structure of these compounds (Figure 1-6) are provided below.

6a: 1-[2-hydroxypropyl-3-(phenylcarbamoyloxy)]-4-phenylpiperazin-1-ium chloride,

6b: 1-[2-hydroxypropyl-3-(phenylcarbamoyloxy)]-(3,4-dichlorophenyl)piperazin-1-ium chloride,

6c: 1-[2-hydroxypropyl-3-(phenylcarbamoyloxy)]-(3-trifluoromethylphenyl)piperazin-1-ium chloride,

6d: 1-[2-hydroxypropyl-3-(phenylcarbamoyloxy)]-4-(3,4-dimethylphenyl)piperazin-1-ium chloride,

6e: 1-[2-hydroxypropyl-3-(phenylcarbamoyloxy)]-4-(diphenylmethyl)piperazin-1-ium chloride,

6g: 1-[2-hydroxypropyl-3-(phenylcarbamoyloxy)]-4-(4-methoxyphenyl)piperazin-1-ium chloride

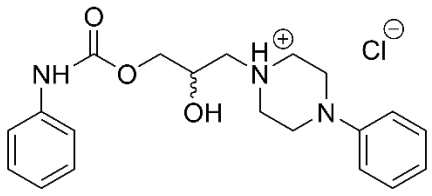

Figure 1. Chemical structure of sample 6a.

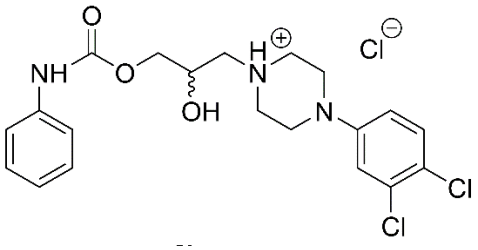

Figure 2. Chemical structure of sample 6b.

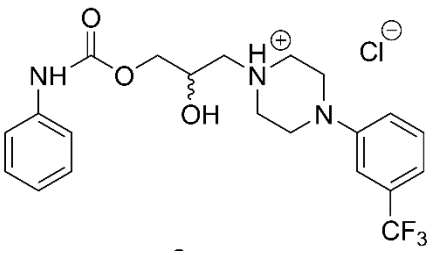

Figure 3. Chemical structure of sample 6c.

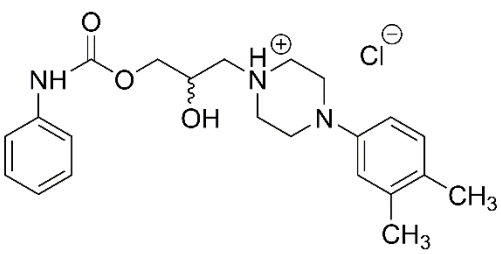

Figure 4. Chemical structure of sample 6d.

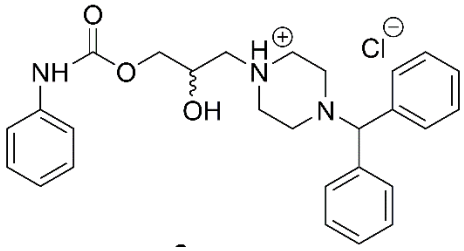

Figure 5. Chemical structure of sample 6e.

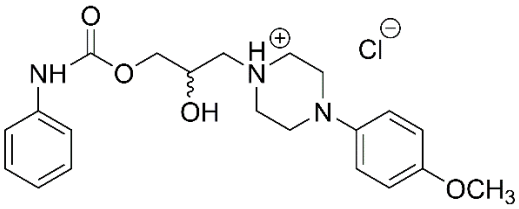

Figure 6. Chemical structure of sample 6g.

Other compounds, which were used in current *in vitro* measurement, were purchased: Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; Fluka Chemie, Switzerland), DPPH radical (2,2-diphenyl-1-picrylhydrazyl; Sigma-Aldrich, Germany) and methanol (Optigrade, Promochem® LGC Standards GmbH, Wesel, Germany). All these chemicals were of an analytical grade. Chemical structure of Trolox (Figure 7a) and DPPH radical (Figure 7b) is provided below.

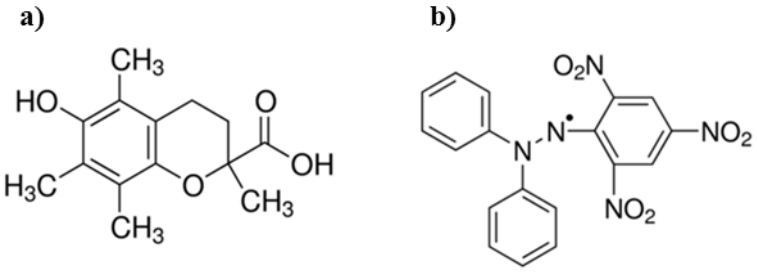

Figure 7a. Chemical structure of Trolox.

Figure 7b. Chemical structure of DPPH radical.

Trolox, a very powerful antioxidant, was used as standard. The DPPH radical is considered the stable one due to its delocalization of a free electron over the molecule. In addition, given radical does not dimerize and has shown strong visible absorption.

METHOD

The ability to reduce the DPPH radical by samples **6a**-**6e** and **6g** was determined with the DPPH assay by following procedure – to 1.8 mL of a DPPH radical methanol solution (1 mg/50 mL), the solution of particular screened compound (sample) dissolved in methanol was added.

In regard to practical arrangement of the experiments, various compounds' concentrations were chosen taking into consideration the increase in mass concentration as well as increase in molar concentration. Thus, the concentrations of compounds **6a**-**6e** and **6g** were as follows: 0.1 g/L, 1.0 g/L, 0.237 mmol/L, and 2.370 mmol/L, respectively. Respective molar concentrations were

calculated from mass concentrations of the sample with the best antioxidant capacity, which was the compound **6g**.

The volume of tested compound's (sample's) solution added to the solution of a DPPH radical (1.8 mL) was 200 μL, or 400 μL. Concentrations and volumes of a Trolox standard solution were the same as concentrations and volumes of compound's solutions. Thus, there were eight sets of measurements together.

The absorbance (*A*) values of a DPPH radical solutions were measured in the UV/Vis area of a spectrum (at a wavelength of λ = 517 nm) after adding the solution of a compound (sample) to the solution of the DPPH radical in the interval of 5, 30, 60, 90, and 120 min, respectively (Table 1, 3, 5, 7, 9, 11, 13, and 15). Those intervals were taken for all measurements *in vitro* and for all relevant calculations, as indicated (Table 1-16). Spectrophotometric device used for the measurements was Biochrom Libra S6 110 (Cambridge, UK).

Antioxidant capacity for each compound (sample) was expressed in percentage of the DPPH radical reduction *(%DPPH* value), which was calculated relatively to the measured *A* of the control according to the equation:

$$\%DPPH = (1 - \frac{A\ sample}{A\ control}) \times 100$$

Control: 1.8 mL of a methanolic DPPH radical solution with added 200 μL, or 400 μL of methanol.

In general, the lower observed *A* value was, the higher *in vitro* antioxidant capacity of particular compound (sample) was. The results are summarized in Table 1, 3, 5, 7, 9, 11, 13, and 15.

RESULTS AND DISCUSSION

Table 1. Absorbance (*A*) values of a mixed DPPH radical solution when the solution of screened synthetic compounds 6a-6e and 6g, or Trolox standard (*c* = 0.1 g/L; addition of 200 µL of particular compound´s solution to the solution of a DPPH radical) was added. The *A* values were measured after 5, 30, 60, 90, and 120 min, respectively.

Addition of a compound	***A* (time; in min)**				
	***A* (5)**	***A* (30)**	***A* (60)**	***A* (90)**	***A* (120)**
6a	0.545	0.545	0.555	0.569	0.584
6b	0.562	0.571	0.585	0.593	0.612
6c	0.560	0.571	0.575	0.592	0.609
6d	0.559	0.566	0.571	0.576	0.588
6e	0.557	0.565	0.570	0.574	0.588
6g	0.536	0.521	0.511	0.507	0.507
Trolox	0.041	0.043	0.047	0.050	0.055
[1]Control	0.570	0.587	0.605	0.622	0.649

[1]Control: 1.8 mL of DPPH radical solution with added 200 µL of methanol

Table 2. Antioxidant potential (*%DPPH*) of the solutions of screened synthetic compounds 6a-6e and 6g as well as Trolox standard (*c* = 0.1 g/L; addition of 200 µL of particular compound´s solution to the solution of a DPPH radical) estimated *in vitro* after 5, 30, 60, 90, and 120 min, respectively.

Compound	***%DPPH* (time; in min)**				
	***%DPPH* (5)**	***%DPPH* (30)**	***%DPPH* (60)**	***%DPPH* (90)**	***%DPPH* (120)**
6a	4.38 %	7.16 %	8.26 %	8.52 %	10.02 %
6b	1.40 %	2.73 %	3.31 %	4.66 %	5.70 %
6c	1.75 %	2.73 %	4.96 %	4.82 %	6.16 %
6d	1.93 %	3.58 %	5.62 %	7.40 %	9.40 %
6e	2.28 %	3.75 %	5.79 %	7.72 %	9.40 %
6g	5.97 %	11.24 %	15.54 %	18.49 %	21.88 %
Trolox	92.81 %	92.68 %	92.23 %	91.96 %	91.53 %

Table 3. Absorbance (*A*) values of a mixed DPPH radical solution when the solution of screened synthetic compounds 6a-6e and 6g, or Trolox standard (*c* = 0.237 mmol/L; addition of 200 µL of particular compound´s solution to the solution of a DPPH radical) was added. The *A* values were measured after 5, 30, 60, 90, and 120 min, respectively.

Addition of a compound	***A* (time; in min)**				
	***A* (5)**	***A* (30)**	***A* (60)**	***A* (90)**	***A* (120)**
6a	0.538	0.540	0.546	0.560	0.575
6b	0.549	0.562	0.577	0.587	0.600
6c	0.549	0.555	0.563	0.577	0.590
6d	0.548	0.554	0.560	0.565	0.577
6e	0.543	0.542	0.549	0.563	0.571
6g	0.532	0.517	0.511	0.509	0.509
Trolox	0.039	0.037	0.034	0.033	0.023
[1]Control	0.558	0.578	0.604	0.646	0.710

[1]Control: 1.8 mL of DPPH radical solution with added 200 µL of methanol

Table 4. Antioxidant potential (*%DPPH*) of the solutions of screened synthetic compounds 6a-6e and 6g as well as Trolox standard (*c* = 0.237 mmol/L; addition of 200 µL of particular compound´s solution to the solution of a DPPH radical) estimated *in vitro* after 5, 30, 60, 90, and 120 min, respectively.

Compound	***%DPPH* (time; in min)**				
	***%DPPH* (5)**	***%DPPH* (30)**	***%DPPH* (60)**	***%DPPH* (90)**	***%DPPH* (120)**
6a	3.58 %	6.57 %	9.60 %	13.31 %	19.01 %
6b	1.61 %	2.77 %	4.47 %	9.13 %	15.49 %
6c	1.61 %	3.98 %	6.79 %	10.68 %	16.90 %
6d	1.79 %	4.15 %	7.28 %	12.54 %	18.73 %
6e	2.69 %	6.23 %	9.11 %	12.85 %	19.58 %
6g	4.66 %	10.55 %	15.40 %	21.21 %	28.31 %
Trolox	93.01 %	93.60 %	94.37 %	94.89 %	96.76 %

Table 5. Absorbance (*A*) values of a mixed DPPH radical solution when the solution of screened synthetic compounds 6a-6e and 6g, or Trolox standard (*c* = 0.1 g/L; addition of 400 µL of particular compound´s solution to the solution of a DPPH radical) was added. The *A* values were measured after 5, 30, 60, 90, and 120 min, respectively.

Addition of a compound	***A* (time; in min)**				
	***A* (5)**	***A* (30)**	***A* (60)**	***A* (90)**	***A* (120)**
6a	0.493	0.477	0.473	0.468	0.463
6b	0.527	0.531	0.540	0.553	0.560
6c	0.531	0.537	0.549	0.560	0.570
6d	0.525	0.528	0.536	0.549	0.549
6e	0.524	0.530	0.547	0.558	0.565
6g	0.471	0.438	0.416	0.402	0.383
Trolox	0.042	0.044	0.041	0.049	0.049
[1]Control	0.524	0.543	0.573	0.604	0.628

[1]Control: 1.8 mL of DPPH radical solution with added 400 µL of methanol

Table 6. Antioxidant potential (*%DPPH*) of the solutions of screened synthetic compounds 6a-6e and 6g as well as Trolox standard (*c* = 0.1 g/L; addition of 400 µL of particular compound´s solution to the solution of a DPPH radical) estimated *in vitro* after 5, 30, 60, 90, and 120 min, respectively.

Compound	***%DPPH* (time; in min)**				
	***%DPPH* (5)**	***%DPPH* (30)**	***%DPPH* (60)**	***%DPPH* (90)**	***%DPPH* (120)**
6a	5.92 %	12.16 %	17.45 %	22.52 %	26.27 %
6b	- 0.57 %	2.21 %	5.76 %	8.44 %	10.83 %
6c	- 1.34 %	1.11 %	4.19 %	7.29 %	9.24 %
6d	- 0.19 %	2.76 %	6.46 %	9.11 %	12.58 %
6e	0.00 %	2.39 %	4.54 %	7.62 %	10.03 %
6g	10.11 %	19.34 %	27.40 %	33.44 %	39.01 %
Trolox	91.99 %	91.90 %	92.85 %	91.89 %	92.20 %

Table 7. Absorbance (*A*) values of a mixed DPPH radical solution when the solution of screened synthetic compounds 6a-6e and 6g, or Trolox standard (*c* = 0.237 mmol/L; addition of 400 µL of particular compound´s solution to the solution of a DPPH radical) was added. The *A* values were measured after 5, 30, 60, 90, and 120 min, respectively.

Addition of a compound	***A* (time; in min)**				
	***A* (5)**	***A* (30)**	***A* (60)**	***A* (90)**	***A* (120)**
6a	0.475	0.480	0.489	0.501	0.516
6b	0.489	0.512	0.518	0.552	0.605
6c	0.495	0.497	0.501	0.518	0.538
6d	0.483	0.492	0.496	0.513	0.528
6e	0.482	0.481	0.491	0.505	0.520
6g	0.460	0.431	0.406	0.405	0.401
Trolox	0.032	0.033	0.025	0.026	0.025
[1]Control	0.504	0.524	0.547	0.575	0.620

[1]Control: 1.8 mL of DPPH radical solution with added 400 µL of methanol

Table 8. Antioxidant potential (*%DPPH*) of the solutions of screened synthetic compounds 6a-6e and 6g as well as Trolox standard (*c* = 0.237 mmol/L; addition of 400 µL of particular compound´s solution to the solution of a DPPH radical) estimated *in vitro* after 5, 30, 60, 90, and 120 min, respectively.

Compound	***%DPPH* (time; in min)**				
	***%DPPH* (5)**	***%DPPH* (30)**	***%DPPH* (60)**	***%DPPH* (90)**	***%DPPH* (120)**
6a	5.75 %	8.40 %	10.60 %	12.87 %	16.77 %
6b	2.98 %	2.29 %	5.30 %	4.00 %	2.42 %
6c	1.79 %	5.15 %	8.41 %	9.91 %	13.23 %
6d	4.17 %	6.11 %	9.32 %	10.78 %	14.84 %
6e	4.37 %	8.21 %	10.24 %	12.17 %	16.13 %
6g	8.73 %	17.75 %	25.78 %	29.57 %	35.32 %
Trolox	93.65 %	93.70 %	95.43 %	95.48 %	95.97 %

Table 9. Absorbance (*A*) values of a mixed DPPH radical solution when the solution of screened synthetic compounds 6a-6e and 6g, or Trolox standard (*c* = 1.0 g/L; addition of 200 µL of particular compound´s solution to the solution of a DPPH radical) was added. The *A* values were measured after 5, 30, 60, 90, and 120 min, respectively.

Addition of a compound	***A* (time; in min)**				
	***A* (5)**	***A* (30)**	***A* (60)**	***A* (90)**	***A* (120)**
6a	0.768	0.551	0.481	0.448	0.428
6b	0.962	0.950	0.982	1.041	1.185
6c	0.950	0.934	0.959	1.027	1.118
6d	0.988	0.960	0.970	0.989	0.950
6e	0.954	0.920	0.912	0.915	0.908
6g	0.682	0.470	0.363	0.316	0.242
Trolox	0.047	0.049	0.051	0.052	0.050
[1]Control	1.015	1.069	1.170	1.263	1.480

[1]Control: 1.8 mL of DPPH radical solution with added 200 µL of methanol

Table 10. Antioxidant potential (*%DPPH*) of the solutions of screened synthetic compounds 6a-6e and 6g as well as Trolox standard (*c* = 1.0 g/L; addition of 200 µL of particular compound´s solution to the solution of a DPPH radical) estimated *in vitro* after 5, 30, 60, 90, and 120 min, respectively.

Compound	***%DPPH* (time; in min)**				
	***%DPPH* (5)**	***%DPPH* (30)**	***%DPPH* (60)**	***%DPPH* (90)**	***%DPPH* (120)**
6a	24.33 %	48.46 %	58.89 %	64.53 %	71.08 %
6b	5.22 %	11.13 %	16.07 %	17.58 %	19.93 %
6c	6.40 %	12.63 %	18.03 %	18.69 %	24.46 %
6d	2.66 %	10.20 %	17.09 %	21.69 %	35.81 %
6e	6.01 %	13.94 %	22.05 %	27.55 %	38.65 %
6g	32.81 %	56.03 %	68.97 %	74.98 %	83.65 %
Trolox	95.37 %	95.42 %	95.64 %	95.88 %	96.62 %

Table 11. Absorbance (*A*) values of a mixed DPPH radical solution when the solution of screened synthetic compounds 6a-6e and 6g, or Trolox standard (*c* = 2.370 mmol/L; addition of 200 µL of particular compound´s solution to the solution of a DPPH radical) was added. The *A* values were measured after 5, 30, 60, 90, and 120 min, respectively.

Addition of a compound	***A* (time; in min)**				
	***A* (5)**	***A* (30)**	***A* (60)**	***A* (90)**	***A* (120)**
6a	0.435	0.346	0.291	0.264	0.251
6b	0.554	0.552	0.569	0.584	0.611
6c	0.565	0.561	0.585	0.619	0.667
6d	0.549	0.547	0.559	0.564	0.576
6e	0.535	0.515	0.510	0.509	0.518
6g	0.373	0.265	0.208	0.178	0.163
Trolox	0.032	0.031	0.033	0.028	0.027
[1]Control	0.561	0.571	0.598	0.623	0.653

[1]Control: 1.8 mL of DPPH radical solution with added 200 µL of methanol

Table 12. Antioxidant potential (*%DPPH*) of the solutions of screened synthetic compounds 6a-6e and 6g as well as Trolox standard (*c* = 2.370 mmol/L; addition of 200 µL of particular compound´s solution to the solution of a DPPH radical) estimated *in vitro* after 5, 30, 60, 90, and 120 min, respectively.

Compound	***%DPPH* (time; in min)**				
	***%DPPH* (5)**	***%DPPH* (30)**	***%DPPH* (60)**	***%DPPH* (90)**	***%DPPH* (120)**
6a	22.46 %	39.41 %	51.34 %	57.62 %	61.56 %
6b	1.25 %	3.33 %	4.85 %	6.27 %	6.43 %
6c	- 0.71 %	1.75 %	2.17 %	0.64 %	- 2.14 %
6d	2.14 %	4.20 %	6.52 %	9.47 %	11.79 %
6e	4.64 %	9.81 %	14.71 %	18.30 %	20.67 %
6g	33.51 %	53.59 %	65.22 %	71.43 %	75.04 %
Trolox	94.30 %	94.57 %	94.48 %	95.51 %	95.87 %

Table 13. Absorbance (*A*) values of a mixed DPPH radical solution when the solution of screened synthetic compounds 6a-6e and 6g, or Trolox standard (*c* = 1.0 g/L; addition of 400 µL of particular compound´s solution to the solution of a DPPH radical) was added. The *A* values were measured after 5, 30, 60, 90, and 120 min, respectively.

Addition of a compound	***A* (time; in min)**				
	***A* (5)**	***A* (30)**	***A* (60)**	***A* (90)**	***A* (120)**
6a	0.340	0.204	0.162	0.148	0.146
6b	0.465	0.471	0.493	0.520	0.559
6c	0.469	0.476	0.498	0.520	0.557
6d	0.462	0.442	0.448	0.462	0.491
6e	0.432	0.371	0.361	0.363	0.380
6g	0.291	0.167	0.132	0.116	0.112
Trolox	0.030	0.030	0.030	0.030	0.033
[1]Control	0.479	0.498	0.530	0.531	0.571

[1]Control: 1.8 mL of DPPH radical solution with added 400 µL of methanol

Table 14. Antioxidant potential (*%DPPH*) of the solutions of screened synthetic compounds 6a-6e and 6g as well as Trolox standard (*c* = 1.0 g/L; addition of 400 µL of particular compound´s solution to the solution of a DPPH radical) estimated *in vitro* after 5, 30, 60, 90, and 120 min, respectively.

Compound	***%DPPH* (time; in min)**				
	***%DPPH* (5)**	***%DPPH* (30)**	***%DPPH* (60)**	***%DPPH* (90)**	***%DPPH* (120)**
6a	29.02 %	59.04 %	69.43 %	72.13 %	74.43 %
6b	2.92 %	5.42 %	6.98 %	2.07 %	2.10 %
6c	2.09 %	4.42 %	6.04 %	2.07 %	2.45 %
6d	3.55 %	11.24 %	15.47 %	12.99 %	14.01 %
6e	9.81 %	25.50 %	31.89 %	31.64 %	33.45 %
6g	39.25 %	66.47 %	75.09 %	78.15 %	80.39 %
Trolox	93.74 %	93.98 %	94.34 %	94.35 %	94.22 %

Table 15. Absorbance (*A*) values of a mixed DPPH radical solution when the solution of screened synthetic compounds 6a-6e and 6g, or Trolox standard (*c* = 2.370 mmol/L; addition of 400 µL of particular compound´s solution to the solution of a DPPH radical) was added. The *A* values were measured after 5, 30, 60, 90, and 120 min, respectively.

Addition of a compound	***A* (time; in min)**				
	***A* (5)**	***A* (30)**	***A* (60)**	***A* (90)**	***A* (120)**
6a	0.371	0.238	0.192	0.171	0.163
6b	0.503	0.501	0.523	0.544	0.581
6c	0.505	0.497	0.509	0.526	0.556
6d	0.498	0.474	0.476	0.478	0.492
6e	0.471	0.393	0.374	0.378	0.402
6g	0.284	0.153	0.112	0.087	0.076
Trolox	0.030	0.029	0.030	0.026	0.026
[1]Control	0.526	0.530	0.550	0.571	0.613

[1]Control: 1.8 mL of DPPH radical solution with added 400 µL of methanol

Table 16. Antioxidant potential (*%DPPH*) of the solutions of screened synthetic compounds 6a-6e and 6g as well as Trolox standard (*c* = 2.370 mmol/L; addition of 400 µL of particular compound´s solution to the solution of a DPPH radical) estimated *in vitro* after 5, 30, 60, 90, and 120 min, respectively.

Compound	***%DPPH* (time; in min)**				
	***%DPPH* (5)**	***%DPPH* (30)**	***%DPPH* (60)**	***%DPPH* (90)**	***%DPPH* (120)**
6a	29.47 %	55.09 %	65.09 %	70.05 %	73.41 %
6b	4.37 %	5.47 %	4.91 %	4.73 %	5.22 %
6c	3.99 %	6.23 %	7.45 %	7.88 %	9.30 %
6d	5.32 %	10.57 %	13.45 %	16.29 %	19.74 %
6e	10.46 %	25.85 %	32.00 %	33.80 %	34.42 %
6g	46.01 %	71.13 %	79.64 %	84.76 %	87.60 %
Trolox	94.30 %	94.53 %	94.55 %	95.45 %	95.76 %

In the current research, antioxidant test *in vitro*, i.e., the DPPH radical reduction assay (Table 2, 4, 6, 10, 12, 14, and 16), was used to preliminary explore eventual differences in antioxidant potency of a series of hybrid synthetic molecules containing a 4-(substituted phenyl)-/4-(diphenylmethyl)piperazin-1-yl structural motif, promising cardiovascular drugs, or antimycobacterial agents in connection with their possible clinical use.

Structural arrangement of screened compounds **6a**-**6e** and **6g**, i.e., the presence of a lipophilic (unsubstituted) aromatic system, polar carbamate moiety, connecting 2-hydroxypropan-1,3-diyl chain and basic moiety (variously substituted cyclic amine), respectively, indicated that they might be used for the treatment of cardiovascular diseases, or infections caused by members of the *Mycobacterium* genus, termed *Mycobacterium tuberculosis* complex, especially *M. tuberculosis* [14-19].

In fact, the free-radical mechanisms have been implicated in the pathology of various human diseases, including cancer, atherosclerosis, malaria, rheumatoid arthritis, or neurodegenerative diseases [14]. Compounds from the class of β-adrenergic receptors antagonists (β-ARAs), which have a very similar (identical) structural pattern, have been primarily used for the treatment of ischaemic heart disease, *angina pectoris*, arrhythmias, hypertension, cardiomyopathy, or as the prevention after myocardial infarction. In addition, those molecules were also accepted as a therapeutic alternative for heart failure [15]. The part of their beneficial cardiovascular effects has been associated with antioxidant properties that some of them have possessed [16].

In 2019, tuberculosis (TB) claimed approximately 1.2 million deaths in HIV-negative individuals and additional 208 000 deaths among people suffering from HIV [20].

The *Mycobacteriaceae* family encompasses a diverse group of bacteria, which show different traits of pathogenicity in animals and humans and exhibits various host reservoirs. The genus of *Mycobacterium* can be classified into two main groups, which includes slow-growing and fast-growing mycobacteria based on their growth rates. For instance, slow-growing mycobacteria consists of *M. bovis*, *M. tuberculosis* and *M. leprae*, which are responsible for bovine tuberculosis, human tuberculosis, and leprosy, respectively, whilst the fast-growing group such as *M. smegmatis* is regarded as an opportunistic or non-pathogenic bacteria in general [19].

During the course of an infection, the *M. tuberculosis* pathogen must cope with a variety of host-mediated stresses, in particular, oxidative and nitrosative stress, or antibacterial properties of macrophages. The macrophages produce Reactive Oxygen Species (ROS) and Reactive

Nitrogen Species (RNS) *via* NADPH oxidase (NOX2/gp^{91phox}) as well as inducible nitric oxide synthase iNOS [21, 22].

High basal resistance of *M. tuberculosis* to the oxidative and nitrosative stress seemed to be a combination of intrinsic resistance of the *M. tuberculosis* cell wall, constitutive expression of genes encoding both ROS and RNS scavenging functions, induction of genes that encoded for repair of oxidized proteins, and induction of DNA repair mechanisms as well [22, 23].

Following the type of substitution within a basic moiety of currently *in vitro* screened set, one can consider the compounds containing electron-donating substituent(s) attached to an aromatic ring (**6d**, **6g**) as promising cardiovascular agents, and the molecules with highly lipophilic substituent(s) showing electron-accepting properties (**6b**, **6c**) promising anti-TB drugs. In addition, both the compounds **6a** and **6e** were chosen for the *in vitro* testing because of their „unsubstituted nature" (**6a**; presence of a 4-phenylpiperazin-1-yl moiety), or in order to indicate the differences resulting from direct attachment of the aromate to a piperazin-1,4-diyl ring, or *via* a hydrophobic methane-1,1-diyl moiety (**6e**). Thus, the consequence of given structural variations might be different antioxidant capacity *in vitro*.

The idea to perform *in vitro* measurements employing various concentrations/volumes of prepared solutions of investigated compounds (samples) was based on the attempt to observe more unambiguous results.

As mentioned, the time to observe eventual DPPH radical reduction based on the activity of tested compounds (samples) was as follows: 5, 30, 60, 90 and 120 min, respectively.

Despite the fact that the compounds **6a**-**6e**, and **6g** contained a stereogenic centre within a connecting chain, they were synthesized and tested *in vitro* as racemates but not pure enantiomers.

The present experimental findings indicated the reference Trolox molecule as the most efficient antioxidant; none of these synthetic hybrid derivatives reached its antioxidant power in order to reduce a DPPH radical. Particular *%DPPH* values for given standard drug were not lower than 91.50 % (Table 1-16).

The research showed that 1-[2-hydroxypropyl-3-(phenylcarbamoyloxy)]-4-(4-methoxyphenyl)piperazin-1-ium chloride (**6g**), 1-[2-hydroxypropyl-3-(phenylcarbamoyloxy)]-4-phenylpiperazin-1-ium chloride (**6a**), 1-[2-hydroxypropyl-3-(phenylcarbamoyloxy)]-4-(diphenylmethyl)piperazin-1-ium chloride (**6e**), and 1-[2-hydroxypropyl-3-(phenylcarbamoyloxy)]-4-(3,4-dimethylphenyl)piperazin-1-ium chloride (**6d**) were the molecules with moderate ability to reduce a DPPH radical; the decrease in their antioxidant efficiency was observed „approximately" in that order.

On the basis of chemical reactions involved, major antioxidant potential assays can be very roughly divided into two categories [24], i.e., hydrogen atom transfer (HAT) reaction based assays, which quantify the hydrogen atom donating capacity, and single electron transfer (ET) reaction based assays, which measure the antioxidant´s reducing capacity.

In other words, an oxidant abstracts electron from an antioxidant causing (color) changes of an oxidant. The DPPH assay was previously believed to involve the HAT reaction, however the papers [25, 26] suggested that given reaction in fact behaved like the ET one.

The Hammett constant σ describes the influence of a functional group on acidity, or basicity of a neighboring site and determines the distribution of partial charges over the surface of a biologically active molecule, modulating its binding behavior towards a target (biological) structure [27]. The more positive the σ value is, the more electron-withdrawing influence of respective atom/group is observed. The σ parameter for a OCH_3 group attached to a 4-position of an aromatic system is –0.27; the H atom is described with $\sigma = 0.00$.

Following the opinion that the reduction of a DPPH radical was the ET reaction, relatively low σ value for a 4-OCH_3 moiety led to the prediction that the radical acting as an oxidant was able to abstract an electron more easily from relatively electron-rich aromatic system of a 4-(4-methoxyphenyl)piperazin-1-yl fragment being included within the molecule **6g** compared to the compounds containing an „unsubstituted“ 4-phenylpiperazin-1-yl moiety (**6a**), or even the substances with electron-accepting substituent(s) attached to the aromate (**6b**, **6c**).

The linearity of a basic fragment in a structure of **6g** made a resonance (mesomeric) effect at a phenyl ring, which affected compound´s distribution of electrons and lipohydrophilic properties as well. The 4-OCH_3 group primarily acted *via* the resonance as an electron-donating fragment, which was able to enhance the basicity of a nitrogen atom of a piperazin-1,4-diyl cycle. Nevertheless, described electron-donating resonance effect was countered by the electron-withdrawing inductive one. Anyway, for a 4-position, a positive mesomeric behavior dominated [28].

The consequence of such structural properties might be recognized in higher *%DPPH* values related to **6g** compared to those entries of **6a**, **6b**, or **6c**.

The capability of the molecule **6g** to reduce most effectively a DPPH radical (excluding the antioxidant power of Trolox) was observed in all measurements regardless of sample´s concentrations, as relevant *%DPPH* values indicated. These parameters were apparently time-dependent, as expected. Elongation of a period for the measurements provided higher *%DPPH* connected with **6g**. The same trend was observed in almost all estimations for the **6a**-**6e** series (Table 2, 4, 6, 8, 10, 12, 14, and 16).

In summary, the molecule **6g** was relatively most promising synthetic antioxidant, which antioxidant feature might be beneficial if it would be an effective cardiovascular agent.

One could be surprised that the „unsubstituted“ derivative **6a** seemed to be more efficient antioxidant than the substance **6d** despite of being characterized by a 3,4-dimethyl substitution at a phenyl ring, or highly lipophilic molecule **6e** containing a 4-diphenylmethyl moiety (Table 2, 4, 6, 8, 10, 12, 14, and 16).

On the other hand, the lowest potential to reduce a DPPH radical was shown by both highly lipophilic 1-[2-hydroxypropyl-3-(phenylcarbamoyloxy)]-(3,4-dichlorophenyl)piperazin-1-ium chloride (**6b**), and 1-[2-hydroxypropyl-3-(phenylcarbamoyloxy)]-(3-trifluoromethylphenyl)piperazin-1-ium chloride (**6c**); the molecule **6c** was only slightly „more efficient“ antioxidant than **6b**.

The summary σ value connected with a 3,4-dichloro substitution of the aromate (**6b**) is +0.60, indicating strong electron-accepting properties of the substituents. In this case, lower capability of the molecule **6b** being oxidized (lower *%DPPH* values) was observed due to the decrease in electron density on the aromatic system.

Similar findings were observed when investigating capability of the analogues containing an 2-/3-alkoxy substituent (alkoxy = methoxy to propoxy) at a phenylcarbamoyloxy moiety to reduce a DPPH radical under conditions *in vitro* [29]. Previous research also pointed that classical bioisosteric replacement of a polar carbamate moiety with an etheric bridge, introduction of an alkoxycarbonylaminophenyl group (alkoxy = methoxy to butoxy) instead on the (substituted) phenylcarbamoyloxy one, and replacement of a basic cyclic amine group with the acyclic one did not provide derivatives with significant improvement in their antioxidant potential [30].

In regard of current research, negative values of the *%DPPH* parameter were found for the compounds **6b** and **6c** in some cases (Table 6, and 12) suggesting their initial pro-oxidant effect. If a synthetic hybrid compound **6b** would effectively fight the *M. tuberculosis* pathogen, one might proposed that its low ability to reduce given RNS, a DPPH radical, could not be probably regarded as the „notable“ obstacle in terms of a primary anti-TB activity.

CONCLUSIONS

When introduced for the first time, ROS and RNS were thought to be very toxic and associated only with various pathological conditions. Since then, a tremendous amount of research has been published connecting ROS and RNS with various physiological processes as well. In this context, the biological role of ROS and RNS is rather complex and paradoxical. There is a close

association between oxidative stress, inflammation and increasing evidence for a causal role of (low-grade) inflammation for the onset and progression of cardiovascular diseases, which may serve as the missing link between oxidative stress and cardiovascular morbidity and mortality. Moderate antioxidant capacity *in vitro* of 1-[2-hydroxypropyl-3-(phenylcarbamoyloxy)]-4-(4-methoxyphenyl)piperazin-1-ium chloride (**6g**) might be a favorable property if focusing on this molecule as an effective cardiovascular agent. On the other hand, relatively low ability of the drugs to reduce, or scavenge ROS and/or RNS could be, paradoxically, beneficial feature in the light of their primary biological (pharmacological) activity. *M. tuberculosis* harbors sophisticated systems to continuously monitor and mount appropriate responses against host generated redox stresses. The pathogen is known to mobilize several transcriptional regulators in response to ROS and RNS. 1-[2-Hydroxypropyl-3-(phenylcarbamoyloxy)]-(3,4-dichlorophenyl)piperazin-1-ium chloride (**6b**) might supposed to be an effective anti-TB agent, as its very suitable structural arrangement and selection of substituents indicated. In addition, the molecule showed the lowest potential to reduce *in vitro* the DPPH radical among the set of currently screened derivatives. Thus, given property would not be considered the „notable" obstacle in terms of (assumed) anti-TB activity.

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