Chapter 1

Introductory Chapter: Unregulated Mitochondrial Production of Reactive Oxygen Species in Testing the Biological Activity of Compounds

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Additional information is available at the end of the chapter

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1. Introduction

Medicinal chemistry is an area that creates important links between the function of living organisms and the action of substances, whether natural or synthetic. This includes studies of structure–activity and dose–response associations in cell culture systems, in vitro and subsequent in vivo studies. The treatment of many diseases requires continuous invention, synthesis, characterization, and final testing of new designed compounds. Recently, there is also growing interest in better and more targeted use of the rich spectrum of effective natural substances extracted from plants. Each study thus contributes to the characterization of the effects of substances in order to achieve the least possible side effects in interactions and metabolism but significant expected ones. Also, part of such studies has been carried out at our workplace, which show the necessary and complementary role of identifying the effects and use of these substances. In this way, we introduce a shortened preview of unregulated endogenous production of reactive oxygen and nitrogen species in the biological activity of compounds in mitochondria.

2. Reactive oxygen species production in mitochondria

Mitochondria are two membrane organelles present in all cells that have a nucleus. They are the energy center of the cells. Their primary role is the production of ATP in oxidative phosphorylation, and the basis of the aerobic oxidation is the citric acid cycle interconnection representing the final metabolic pathway of oxidation of all major nutrients to the respiratory

chain where oxidation of reduced coenzymes results in ATP formation. By the process of oxidative phosphorylation, the mitochondria have an irreplaceable function in the formation of metabolic energy in the form of ATP. The electrodes released in this process from reduced substrates are transferred to O_2 via the H^+ pumps of the respiratory chain. Pumps (complexes I–IV) form a H^+ gradient through the internal mitochondrial membrane, and the electrochemical energy of this gradient is then used to synthesize ATP complex V, ATP synthase [1]. Gradual reduction of O_2 occurs through several interstages when reactive oxygen species (ROS) are formed. One-electron reduction of O_2 to superoxide radical ($O_2^{\bullet}-$) is thermodynamically more advantageous, even for substances with relative oxidation ability, so in the mitochondria, a number of electron donors potentially allow this reaction [2]. However, only a small number of mitochondrial electron transporters with thermodynamic potential to reduce O_2 actually act. In most cases, small-molecule electron transporters such as NADH, NADPH, reduced coenzyme ($CoQH_2$), and reduced glutathione (GSH) do not react with O_2 but regenerate it. Instead, $O_2^{\bullet}-$ production takes place on the redox-active prosthetic groups of proteins or electron-binding proteins such as $CoQH_2$, which is a kinetic factor that allows or prevents the reduction of O_2 molecules and determines the production of $O_2^{\bullet}-$ in the mitochondria [3]. The mechanism of mitochondrial production and release of H_2O_2 and $O_2^{\bullet}-$ can be seen as described in more detail in [4]. Overall, in aerobic metabolism, the mitochondrial oxidative phosphorylation system balances the reduction of O_2 to H_2O in maximizing ATP synthesis with the simultaneous production of ROS only to the amount required for cell signaling [5].

The major part of the mitochondrial ROS production is formed as a by-product of the respiration on the inner side of the inner mitochondrial membrane. Complex IV (cytochrome c oxidase), a terminal component of the electron transport chain, receives four electrons from cytochrome c and reduces one molecule of O_2 to two H_2O. The intermediates remain partially reduced until they are completely reduced and are not secreted in measurable amounts [6]. Historically, the $O_2^{\bullet}-$ producing complex III was described as the first site of ROS production—in the Q-cycle [7]. Theoretically, the oxidation of succinate by succinate dehydrogenase (complex II, SDH) leads to significant $O_2^{\bullet}-$ formation—but so far it has not been measured. Thus, it is not entirely clear whether SDH produces in situ mitochondrial ROS. Nevertheless, the production of ROS complex II is a significant source in many tissues via the reverse electron transport mechanism. This particular phenomenon results from a high membrane potential that thermodynamically favors complex II as a donor for complex I., thanks to which succinate supports production of ROS in complex I. Thanks to which succinate supports production of ROS in complex I [6]. Complex I (NADH dehydrogenase) is the main entry of electrons into the respiratory chain. It is a significant source of ROS, namely, $O_2^{\bullet}-$ and H_2O_2, although it is very complicated to find out whether it is the major source of ROS in mitochondria in vivo. All evidence of significant ROS production was obtained in in vitro studies [8].

The mitochondria also contain other sources, outside the respiratory chain that highlight ROS production. On the outer mitochondrial membrane, there are cytochrome b5 reductase and monoamine oxidase. Cytochrome b5 reductase is present in all mammalian tissues and is capable of production $O_2^{\bullet}-$ at a very high rate of about 300 $mmol.min^{-1}.mg$ of $protein^{-1}$. Monoamine oxidases (MAO-A and MAO-B) are also present in all tissues of mammals.

They catalyze the oxidation of biogenic amines while releasing H_2O_2 [9]. On the outside of the internal mitochondrial membrane, the conversion of dihydroorotate to orotate catalyzes the de novo synthesis of uridine monophosphate dihydroorotate dehydrogenase. It is considered to be the source of $O_2^{\cdot}-$ and H_2O_2, although its ability to produce $O_2^{\cdot}-$ requires further study. Also, part of a glycerophosphate shuttle, the mitochondrial glycerol-3-phosphate dehydrogenase, is present in all cells but with uneven expression and mediating the formation of H_2O_2 [10]. In the mitochondrial matrix, there is localized aconitase catalyzing the conversion of citrate to isocitrate within the Krebs cycle. Enzyme contains a Fe-S cluster that can be oxidized by $O_2^{\cdot}-$ but also H_2O_2 leading to $^{\cdot}OH$ production [11]. The subunit of lipoamide dehydrogenase from the ketoglutarate dehydrogenase complex, located on the inner side of the membrane turned into the mitochondrial matrix, produces $O_2^{\cdot}-$ and H_2O_2. The subunit of the pyruvate dehydrogenase complex, dihydrolipoyl dehydrogenase, is also a significant source of ROS [12].

Mitochondria and ROS signaling control cell homeostasis by regulating processes of physiological cell death (apoptosis), including autophagy however also that of survival. Damage of mtDNA, protein carbonylation, or lipid peroxidation due to increased ROS production have been documented in many studies, and due to the localization and metabolic role of the organelles, they can lead to an energy disaster of the cell. Therefore, the production of ROS by mitochondria is considered crucial for cell survival or death. Many proteins that mediate apoptosis and autophagy directly affect ROS signaling by translocation into the mitochondria compartment and subsequent modulation of pro- or antioxidant enzymes [9].

3. Testing compounds

Synthetic and natural substances can affect the production of ROS; alter the redox state of the cell and, depending on the extent of the oxidative change; affect proliferation; or induce apoptosis. Chalcones are intermediate products of biosynthesis of a wide variety of plant polyphenols, flavonoids. Chalcones, as α, β-unsaturated carbonyl compounds have a wide range of substituents. The cycles are connected by three strongly electrophilic carbons, and the whole system creates a linear or almost planar structure [13]. They also contain a keto-ethylene group (–CO–CH=CH–). They have conjugated double bonds and a fully delocalized π-electron system on both benzene rings [14]. Structure–activity studies have shown that the cytotoxicity of chiral analogues is affected by the shape of the molecules [15, 16]. The multimodal pharmacodynamic, structural diversity of synthetic and natural chalcones and the constitutive elements that create optimal toxicity vary for each class of chalcones, and there are no generally valid rules of relationship between structure and activity [17]. Changes in the structure create a high degree of diversity which, as was shown, is useful for the development of new drugs with better efficacy, lower toxicity, and good pharmacological action. Thus, chalcones have become of interest not only in the academic but also in the industrial sphere and are used as intermediates for the preparation of compounds having therapeutic utility. They are currently used in the treatment of viral, cardiovascular diseases, parasitic infections, pain, inflammation, and gastric cancer, as well as additives and cosmetic ingredients [14].

Some natural but also synthetic chalcones have demonstrated cytotoxic activity against tumor cell cultures by inhibiting cell growth. However, they are also effective as anticancer and chemopreventive agents in vivo [18–20]. The amount of clinically useful antitumor drugs exhibits a genotoxic effect based on their affinity to amino groups of nucleic acid, but chalcones exhibit a pronounced affinity to thiols [15]. These reactions can alter intracellular redox (redox signaling) that can modulate processes such as DNA synthesis, enzyme activation, selective gene expression, and cell cycle regulation [21]. Many of the pharmacological potentials of chalcones are not used yet.

Summarizing the current knowledge of chalcone efficiency and their cyclic analogues ((*E*)-2-arylmethylene-1-indanone, (E)-2-arylmethylene-1-tetralone, and (*E*)-2-arylmethylene-benzosuberone) with several types of substituents, our studies were then primarily focused on monitoring their effects on mitochondria with respect to the production of ROS and the subsequent effects on selected antioxidant markers and ATP production. As the primary organ of xenobiotic metabolism in the body is the liver, studies with 4′-methyl-, methoxy- [16], 4′-hydroxy- [22], and 4′-dimethylamino-cyclic analogues of chalcones [23] were provided on mitochondria isolated from the rat liver. Analogues with methyl substituents showed rather a protective, antioxidant effect. Observed insufficiency in the antioxidant system and the level of reduced glutathione and associated enzymes such as glutathione peroxidase and glutathione reductase were significantly induced by the presence of benzosuberone in all types of substituents. They act as uncouplers of mitochondrial respiration, thus reducing ATP production. 4′-Hydroxy and 4′-dimethylamino analogues of chalcones exhibited similarly toxic effects as (*E*)-2-arylidene-1-indanones. Chalcones with substituents that increase the electron density of the B-ring, such as methoxy, butoxy, or dimethylamino groups, do not exhibit significant reactivity to reactive species [24].

The current level of knowledge makes it possible to use some of these biological properties of chalcone derivatives influenced by the nature of their substitution, such as the ability to inhibit 12-lipoxygenase and cyclooxygenases with 2′-hydroxychalcones, 4′-hydroxychalcones, and 2′,4′-dihydroxychalcones. Selective inhibitory effects on arachidonic-induced platelet aggregation predict them as antithrombotic or anti-inflammatory agents [25]. Under the low pH, the amino group, which are conditions normally found in tumors, is in protonated form increasing β-carbon electrophilicity in enone linkage, thereby increasing its reactivity as nucleophile acceptor in Michael additions [15], for example, thiol groups. Substantial antiproliferative activity was observed for chalcones with substituted amino groups [26]. All benzosuberone cyclic analogues at incubation with mitochondria caused a significant decrease in reduced glutathione (GSH) levels and simultaneous increase in glutathione peroxidase (GPx) activities. Lowering GSH levels most clearly defines the conditions of strong oxidative stress and leads to changes in the redox potential of the cell [27]. Although many antioxidant defense systems exist in the mitochondria, their maintenance is energy demanding. The first condition is a sufficient amount of ATP needed to synthesize low molecular weight antioxidants and molecules that provide uptake of ROS and ROS by-products. Benzosuberone as well as indanone analogues acted in mitochondria as phosphorylation deactivators, thereby reducing ATP production. GSH itself is able to reduce reactive oxygen and nitrogen species (RNOS); however, it is synthesized only in cytosol. Although it easily passes through the outer mitochondrial membrane via porin

channels, it cannot pass through the inner mitochondrial membrane into the matrix as the anion. Here, the 2-oxoglutarate antiport is applied [28]. By importing GSH, mitochondria lose an important intermediate of the Krebs cycle, which must be replaced by anaplerotic reactions. It is important to note that in experiments, we have been working with isolated mitochondria where transfer of de novo synthesized glutathione was not possible. However, the energy intensity to maintain the redox status was not reduced. $O_2^{\cdot}-$ produced in the mitochondrial matrix, the membrane space, and the outer mitochondrial membrane reacts with other electron acceptors such as NO but primarily leads to the production of H_2O_2. The local antioxidant capacity of peroxidases then determines oxidative damage or H_2O_2-mediated signaling. The GPx catalytic mechanism requires cyclic oxidation/reduction of cysteine or selenocysteine residues at the catalytic center where GSH is used as a cofactor and GSSG is formed. Reactivation through glutathione reductase (GR) requires a reduction potential of NADPH, whose production also requires energy. The presence of glucose-6-phosphate dehydrogenase (and also isocitrate dehydrogenase), a source of NADPH formation in mitochondria, have been proven [29], but the enzyme is extremely sensitive to H_2O_2 levels, leading to its inhibition [29]. Affection of ATP production and changes in GSH levels are associated with the affection of apoptosis, cell division, and growth [30]. According to the decrease in mitochondrial membrane potential, they are able to incorporate into the membrane and induce apoptosis [31]. Conversely, the reduction of ROS production in the mitochondria by partial uncoupling of oxidative phosphorylation, such as observed with tetralone analogues, is a protective mechanism and also corresponds to the measured values of antioxidant parameters.

4. Conclusion

As a result of our observations, (E)-2-arylidene-1-tetralone shows antioxidant and (E)-2-arylidene-1-benzosuberone significant pro-oxidant and cytotoxic properties regardless of the character of the substituent. The findings have contributed to the targeted synthesis of derivatives that are expected to enhance the effect due to structural modification. The mentioned cyclic analogues of chalcones served as a structural substrate, with the aromatic ring B being replaced by a ferrocene. In previous studies on various different ferrocene derivatives, some have been shown to exhibit surprisingly high toxicity and antiproliferative activity [32–34]. Among the unique derivatives available for study, the antiproliferative effect of the (E)-3-(ferroceneethylene)-4-chromanone was the most effective [35]. The pronounced effect of cell viability and colony formation of cancer cells in dose-dependent manner has been shown by 1,1′-bis[(1-oxoindane-2-ylidene) methyl] ferrocene. The mechanism by which tested ferrocenyl compounds could demonstrate these remarkable properties is probably based on their activity with RNOS, which subsequently affected the antioxidant mechanisms of mitochondria. In general, the activity of the compounds with respect to $^{\cdot}OH$ and nitric oxide (NO) was very weak but, however, marked toward $O_2^{\cdot}-$ and the peroxynitrite anion (ONO_2-). The 1,1′-bis[(1-oxoindane-2-ylidene) methyl]ferrocene and 1,1′-bis[(1-oxotetralin-2-ylidene) methyl] ferrocene were involved in the significant production of $O_2^{\cdot}-$, leading to increased activities of superoxide dismutase; additionally, 1,1′-bis[(1-oxoindan-2-ylidene)methyl] ferrocene also exhibited the lowest inhibition of NO and ONO_2-. In addition to preserved

concentrations of GSH, the mechanism of action, especially in this most effective derivative, is likely to be the modulation of mitochondrial activity through the induction of nitrosative stress.

The demonstration from our workplace suggests a long-term process of characterizing the effects of compounds, which contributes only a small amount to their complex knowledge. That is why authors have been invited to create the content of this book to bring their work and theoretical experiences in this area through their chapters.

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