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Lipocarbazole, an efficient lipid peroxidation inhibitor anchored in the membrane

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ABSTRACT

Lipid peroxidation is a major deleterious effect caused by oxidative stress. It is involved in various diseases such as atherosclerosis, rheumatoid arthritis and neurodegenerative diseases. In order to inhibit lipid peroxidation, antioxidants must efficiently scavenge free radicals and penetrate inside biological membranes. Lipocarbazole has recently been shown to be a powerful antioxidant in solution. Here, we show its powerful capacity as lipid peroxidation inhibitor. Its mechanism of action is rationalized based on molecular dynamics simulations on a biomembrane model, quantum calculations and experimental evaluation. The role of the lipocarbazole side chain is particularly highlighted as a critical chemical feature responsible for its antioxidant activity.

1. Introduction

Oxidative stress is defined as an imbalance between production and regulation of reactive oxygen species (ROS), mainly free radicals. The subsequent ROS overproduction can be induced by many factors including UV light, hypoxia, cytokines, chemotherapy and high-energy radiation.[1] Various endogenous antioxidant systems regulate ROS production, namely enzymes (e.g., superoxide dismutase, glutathione peroxidase, and catalase) and small compounds (e.g., glutathione). Exogenous antioxidants, contained in food (e.g., vitamins C and E, polyphenols, carotenoids) or food supplementation, also contribute to the total antioxidant action. Long-term effects of oxidative stress have extensively been studied over the past years; they have been shown to be responsible for various diseases, for example, cardiovascular, Alzheimer and liver diseases.[2] In this context, lipid peroxidation (LPO) is one of the most important processes involving free radicals and it is directly implicated in various diseases such as atherosclerosis, rheumatoid arthritis and neurodegenerative diseases.[3,4] To discover new LPO inhibitors is of particular importance in order to prevent those diseases. From a clinical point of view extensive research deals with new antioxidants being able to decrease lesions induced by ischemia/reperfusion in organ transplantation.[5] It is also a challenge of major importance in cosmetics and food industries.

A series of lipocarbazole derivatives was isolated from the bacterium *Tsukamurella pseudospumae* Acta 1857.[6] These compounds were later synthetized by a series of metal-catalyzed reactions.[7] Due to their structural analogy with carazostatin,[8] an effective in vivo antioxidant, the antioxidant capacity of lipocarbazole is under scrutiny in this article. It was found that lipocarbazole A3 (**1**) (Fig. 1) is more active than ascorbic acid at scavenging DPPH (1,1-diphenyl-2-picrylhydrazyl) in methanol.[6] The DPPH assay is extensively used to provide a solid starting point to evaluate the capacity of a compound at scavenging free radicals by hydrogen and electron transfers.[9] An effective LPO inhibitor must (i) scavenge efficiently free radicals from both thermodynamic and kinetic points-of-view, which is indeed well-related to free radical scavenging and (ii) incorporate into lipid bilayer membranes. The combination of both features allows the compound to efficiently inhibit the LPO chain reaction.[4]

Molecular modeling is a unique and powerful tool allowing the evaluation of these two properties at the atomic scale. In the present study, the thermodynamics of free radical scavenging reactions was obtained using quantum chemistry calculations for compound **1**, whereas molecular dynamics (MD) simulations was

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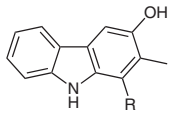

Figure 1. Chemical structures of lipocarbazole derivatives. 1: R = $(CH_2)_7CH{=}CH(CH_2)_7CH_3$. 2: 'carbazole': R = H. 3: carazostatin: R = n-C_7H_{15}.

performed to describe its incorporation, position and orientation into a lipid bilayer model. In order to evaluate the role of the lipid side chain, MD simulations (5 μs total) were performed for **1** and its lipid-side-chain-less counterpart derivative, hereafter referred as 'carbazole' (**2**).

2. Material and methods

2.1. Preparation of Large Unilamellar Vesicles and lipid peroxidation inhibition

A solution (100 μL) of L-α-phosphatidylcholine from soybean (95%) at 40 mg/ml (Soy-PC, Avanti® Polar Lipids inc.) was prepared in chloroform and was further evaporated under vacuum in a round bottom flask to produce thin Soy-PC film. Multilamellar vesicles (MLV) were produced by vortexing the thin film after hydration with distilled water. The MLVs were extruded through a 0.1 μm double layer polycarbonate membrane using a Lipex™ extruder (Northern Lipids) to produce Large Unilamellar Vesicles (LUV). The preparation resulted in a aqueous solution of $2.50 \cdot 10^{-4}$ M Soy-PC LUV. The particle size was ranging from 90 to 110 nm, as determined using an N4plus submicron particle size analyzer (Beckman-Coulter).

The tested compounds of various concentrations (6.2 μL in methanol) were added to 500 μL of LUVs mixture prior to the lipid peroxidation initiation. Oxidative stress was generated by peroxyl radicals (R-OO·) produced during AAPH (2,2′-azobis (2-methylpropionamidine) dihydrochloride, Aldrich) degradation for 90 min at 37 °C (125 μL, 2.5 mM in water). The oxidative stress effect was determined following the formation of conjugated dienes at λ = 233 nm UV–visible absorption (Shimadzu UV-2401PC).[10]

2.2. Bond dissociation enthalpies

Over the past decade, free radical scavenging by polyphenols have been extensively investigated using quantum chemistry calculations.[11–14] The O–H bond dissociation enthalpy (BDE) was shown to be the major descriptor to predict free radical scavenging; the lower the BDE, the higher the capacity of H-atom transfer (HAT) from the antioxidant to the free radical, and the higher the antioxidant activity of the corresponding OH group. It perfectly and systematically correlates with DPPH scavenging. It is a thermodynamic intrinsic parameter calculated for all potentially labile chemical groups (mainly OH groups here) as the following difference in enthalpy (at 298 K):

$$\mathrm{BDE}(Antiox-\mathbf{H}) = \mathrm{H}^{298\mathrm{K}}(Antiox^{\cdot}) + \mathrm{H}^{298\mathrm{K}}(\mathbf{H}^{\cdot}) - \mathrm{H}^{298\mathrm{K}}(Antiox-\mathbf{H}), \quad (1)$$

$\mathrm{H}^{298\mathrm{K}}$(*Antiox*-**H**) being the enthalpy of the antioxidant and $\mathrm{H}^{298\mathrm{K}}$(*Antiox*·) being the enthalpy of the radical formed after H atom abstraction.

Flavonoid derivatives and their corresponding aryloxyl radicals were found to be accurately described by density functional theory (DFT) calculations.[14] The B3P86 functional has been shown to be particularly well-adapted to evaluate the thermodynamics of the reaction between polyphenols and free radicals.[12,14,15] The 6-31+G(d,p) basis set is used since it provides very similar results compared to the larger and more computationally demanding 6-311+G(2d,3pd) basis set;[15] in particular the use of triple-ζ basis sets and the second diffuse function did not significantly enhance BDE predictions (difference lower than 1 kcal mol^{-1}). Geometries, energies including the zero-point correction (V) and enthalpies (H) at 298 K were determined at the (U)B3P86/6-31+G(d,p) level. Ground-state geometries were confirmed by a vibrational frequency analysis that indicated the absence of imaginary frequency.

The solvent effect was taken into account using the integral-equation-formalism polarizable continuum model (IEF-PCM) as implemented in Gaussian 09.[16] Continuum models consider the molecular system embedded in a shape-adapted cavity surrounded by a dielectric continuum characterized by its permittivity (for water ε = 78.4). Calculations in water reproduce a polar physiological environment, while calculations in the gas phase and in benzene give a good approximation of non-polar conditions such as lipophilic membranes. The implicit solvent model weakly influences the quantitative evaluation of phenolic BDE values but may slightly alter qualitative description, that is, modifying the relative contribution of the different H atom donor groups.[12] All calculations were carried out by the Gaussian 09 software.[16]

2.3. Force field and membrane model

All MD simulations were carried out using the GROMACS package version 4.5.4.[17] Two compatible united-atom force fields were used, namely GROMOS 53a6[18] and Berger's[19] for water/hetero-molecules and phospholipids, respectively. The model of bilayer membrane consisted of 128 molecules of 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC)[20] surrounded by approximately 5400 water molecules (SPC/E model). Phosphatidylcholines represent the main type of phospholipids in human membranes.[21] The Na^+ and Cl^- ions were added to the system by replacing water molecules using the Genion program, according to a regular physiological concentration C (0.9% = 0.154 mol L^{-1}). Since Genion calculates the number of ions to be added according to the volume of solvent first obtained as the box volume. Since membrane is empty of water molecules, the corrected concentration C_{corr} was re-calculated as:

$$C_{corr} = C.\frac{z_{box} - z_{membrane}}{z_{box}} \quad (2)$$

2.4. Solute parameters

For the solutes (carbazole and lipocarbazole derivatives), the topologies were obtained from the PRODRG2 webserver.[22]

The partial charges defined by PRODRG2 webserver were significantly lower than those issued from the GROMOS force field and were shown to describe poorly the partitioning between aqueous and cyclohexane phases.[23] The restrained fit of electrostatic potential (RESP)[24] partial charges were alternatively used. RESP-type charges were successfully used in lipid bilayer simulations of several compounds.[25–29] The ESP charges were obtained from B3LYP/aug-cc-pVTZ[30] calculations obtained on geometries optimized at the same level, with Gaussian 09 software.[16] RESP fit was carried out with the Antechamber package of AMBER 11.[31]

2.5. Free MD simulations

Several free simulations were carried out for every studied molecule, with different starting points (far from, close to and inside the lipid bilayer membrane). Energy minimization using the steepest-descent algorithm was performed before production simulations, that is, hundreds ns long MD simulations. The Leap-frog Verlet integrator was used with a 2 fs time step. The cut-off