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Interaction studies between human α-tocopherol transfer protein and nitric oxide donor tocopherol analogues with LDL-protective activity

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ABSTRACT

Nitric oxide-releasing α-tocopherol mimetics with LDL-protective activity were designed to maintain the tocopherol substructure necessary for its biochemical recognition by α-tocopherol transfer protein. In order to study the molecular interactions to α-TTP, theoretical binding studies by means of docking techniques and experimental binding assays, using a fluorescent probe, were performed. Furoxanyl-tocopherol-hybrid analogs **7** and **9** have the best ability to bind to α-TTP suggesting that they could be incorporated to LDL in vivo to further release nitric oxide and prevent oxidative modifications.

1. Introduction

Atherosclerosis and its cardiovascular complications constitute the major cause of morbidity and mortality in developing countries.[1] Although the mechanism underlying the development of atherosclerosis is complex and multifactorial[1,2] in the past decades, a series of significant studies were documented suggesting that vascular oxidative stress, mainly when it is associated with modification and oxidation of low density lipoproteins (LDL), plays a crucial role in atherogenesis.[3] Hence, antioxidant supplementation might prevent/retard the development of atherosclerotic lesions. Despite the fact that known antioxidant and biological properties of vitamin E could account for effective protection, as shown by several clinical and experimental studies, its efficacy remains controversial in the light of some recent clinical trials and *meta*-analyzes.[4–6] However, several factors could explain this failure: criteria for selection of patients, relatively short duration and suboptimal dosages of vitamin E treatment, and poor patient compliance added to the lack of monitoring of vitamin E levels.[6] In any case, the development of novel drugs with improved in vivo antioxidant capacities continues being a necessity for atherosclerosis therapy.[7] On these bases we have recently designed, synthesized, physicochemically and biologically characterized a large series of hybrid molecules combining α-tocopherol (α-T) substructures and nitric oxide (·NO) releasing moieties (i.e., organic nitrates and furoxanyl, Chart 1).[8–11] ·NO is a free radical species that has strong biological antioxidant properties. For example, ·NO inhibits LDL oxidation by scavenging lipid propagatory radicals[12,13] and it is capable of diffusing into LDL where it is a more effective lipid antioxidant than α-T.[14,15] α-T, the major form of vitamin E in plasma, is selectively targeted into LDL during its metabolism mostly due to its binding to α-TTP (α-tocopherol transfer protein) in the liver. This protein selectively sorts out α-T from all incoming tocopherols for incorporation into very low density lipoprotein which are hydrolyzed by endothelial lipoprotein lipase and converted to LDL, the major carrier of α-T to the peripheral tissues. Therefore, the previously developed hybrid compounds could be directed to LDL, if α-TTP could act as its transporter, and consequently trigger ·NO specifically at this site, protecting LDL from oxidative modifications and thus being useful for the prevention of cardiovascular diseases.

In light of the above mentioned background, herein we analyzed the affinity of hybrid α-T-·NO releasing compounds to the α-T transporter protein, α-TTP, in terms of theoretical (docking techniques) and experimental (using a fluorescent tocopherol analog) binding studies. Both studies demonstrated that the compounds were able to bind to α-TTP especially furoxanyl-tocopherol hybrid analogs **7** and **9** (Chart 1). Both compounds bind to α-TTP as well as α-T, suggesting that they could be incorporated into LDL particles in vivo

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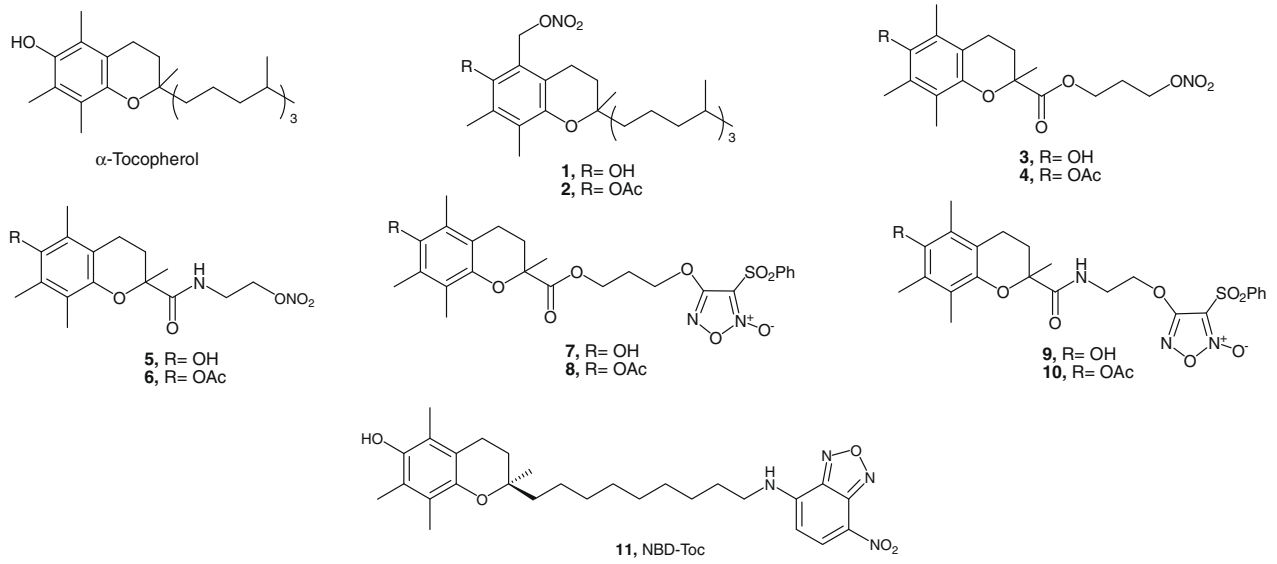

Chart 1. Chemical structures of α-tocopherol, hybrid a-T-·NO releasing compounds (**1**–**10**) and fluorescent tocopherol analog used as probe in the α-TTP-assays (**11**).

using this system. In addition, docking studies allow us to analyze these interactions and explain our experimental results. This could be used in the design of novel non-natural antioxidants with better binding properties to TTP, and thus with improved antioxidant properties in vivo.

2. Results and discussion

2.1. Chemistry

The studied compounds were previously designed and synthesized[8–10] following structural criteria. It is well known that α-T distribution to tissues by the action of α-TTP and other α-T binding proteins is governed by the recognition of certain structural features: a fully methylated chroman ring, a phytyl tail, and *R*-configuration at C-2 where the tail attaches to the chromanol ring. It has been suggested that this recognition plays an important role in its incorporation into LDL particles[16,17] but the protection of isolated LDL from oxidation was increased with decreasing the isoprenoid length side chain at 2-position.[7,18,19] Consequently, we designed structures that sustain pharmacophores of recognized ·NO releasing capability, organic nitrates and furoxans, coupled through appropriate spacers to the chroman ring at the 2- and 5-positions. The selected hybrid compounds for the current study were chosen taking into account their physicochemical and biological properties.[8–10]

2.2. Docking studies

In order to study the molecular interactions of the developed hybrid compounds (Chart 1) to α-TTP, we performed theoretical binding studies by docking techniques. The 3D complex structure of α-TTP with α-T has been solved using X-ray diffraction (Protein Data Bank, www.pdb.org, PDB code 1oip).[20] Thus, it is possible to use this complex as a template to construct the initial complexes. The analysis of the structure of α-TTP with α-T shows that the most of the interactions between α-TTP and α-T are due to van der Waals contacts, but there are three well-ordered water molecules that form a hydrogen-bonding network in the ligand-pocket (see schematic representation in Fig. 1). One of the water molecules connects the tocopherol phenolic hydroxyl group with the backbone carbonyl groups of Val182 and Leu189 through hydrogen

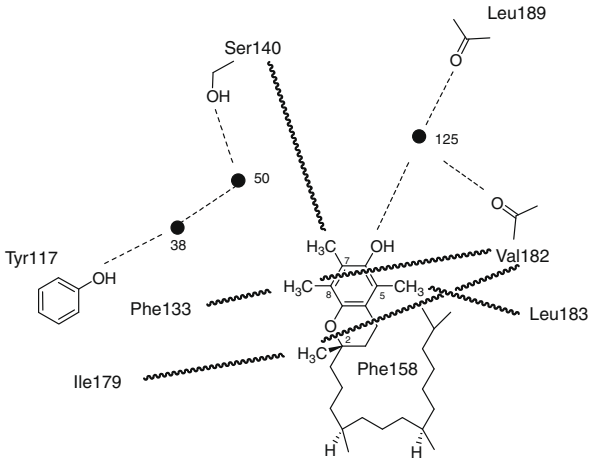

Figure 1. Schematic drawing of the interactions between *R*,*R*,*R*-α-T and the α-TTP residues within the tocopherol-binding pocket. Amino acids that form van der Waals contacts with α-T or participate in hydrogen-bonding networks in the ligand-binding pocket are shown. Active site waters of solvation are represented as black dots (**38**, **50** and **125**).

bonds. Another water molecule participates in a hydrogen bond with the hydroxyl group of Ser140 side chain and the third water molecule is also bounded to Tyr117 through a hydrogen bond. A fourth water molecule is near the phytyl tail but it is not closely associated with α-T. Van der Waals contacts mainly implicate α-T-methyl groups in the 2-position (Val182, Ile179), 5-position (Leu183), 7-position (Ser140), 8-position (Val182, Phe133) and the phytyl side chain (Phe158).[20,26]

Thus, using the complex structure of α-TTP with bound α-T as template we constructed the initial α-TTP/synthesized compounds (ligand) complexes **1–10** (all derivatives as 2-*R*-enantiomers, Chart 1). These initial complexes were minimized using the MMFF94 force field and charges.[21–23] These complexes were the input structure for docking using FLEXIDOCK command implemented in the molecular modeling package SYBYL 7.2.[24,25] FLEXIDOCK software is a program of flexible docking which explore possible orientations of ligand into the protein active site using genetic algorithms. Thus, the program analyzes all possible conformations of the ligand binding the receptor active site, modifying both receptor side

chains and ligand conformations. The final solutions that provide the FLEXIDOCK program were analyzed taking into account mainly the relative energy values. Moreover, the more relevant interactions of tocopherol with α-TTP in X-ray structure[20] was also considered into the binding site with the corresponding aminoacidic motives (Leu 183, Val 182, Ile 179, Phe 158, Ser140, Phe 133). This analysis of the enzyme/ligand complexes generated after docking was based on the distances from the binding site, the hydrogen bond interactions, aromatic and hydrophobic interactions calculated with LPC program. The best scored complex was energy minimized until gradient 0.01 kcal mol^{-1} $Å^{-1}$ using the above mentioned conditions. The binding free energies (ΔG_{bind}) of all complexes were calculated with Structural Thermodynamics Calculations V4.3 (STC) program[27] in order to predict the biological activity of the ligands. STC program calculates the ΔG_{bind} from a parameterization (per $Å^2$ of polar and nonpolar accessible surface area to solvent, ASA) of the heat capacity, enthalpy, and solvation entropy obtained from a global fit of structural and thermodynamic database of globular proteins. The ΔG_{bind} values of the complexes are gathered in Table 1. These data indicate a favorable interaction of derivatives **1**, **2**, **7** and **9** in the binding site, although derivatives **1** and **2** appears to have the best binding free energy. On the other hand, the energy of binding for acetyl derivatives **8** and **10** calculated with the STC program provides a positive value. The volume of derivatives **8** and **10** prevent any possible interaction favorable with α-tocopherol into the binding site.

Employing the STC program we calculated the ASA of protein aminoacid residues of the free protein and the corresponding complexes in order to identify the residues involved in the binding site, those residues that show an important change in the surface area accessible to solvent are involved in some type of interaction, therefore these residues are forming the binding site. In addition it is possible to quantify the contribution of each amino acid to the binding process. Those residues that showed relevant changes regarding ASA have been collected in Table 2 with their partial contribution to the binding energy to the complex (ΔG_{bind}). All derivatives (**1–7** and **9**) establish significant interactions with residues Phe158, Ile179 and Val182. Furthermore, there is also interaction with Phe133, Ser140 and Leu183. In the case of compounds **7** and **9**, it is worth mentioning the contribution of Tyr100 to the ΔG. In addition there are also significant interactions with Val 132 and Ser 136.

Table 1
ΔG_{bind} and K_d of ligands

Complex	ΔG (kcal/mol)	K_d (M)
α-T	−7.66	2.42×10^{-6}
1	−8.11	1.13×10^{-6}
2	−8.79	0.36×10^{-6}
3	−5.16	1.65×10^{-4}
4	−6.00	0.40×10^{-4}
5	−5.41	1.08×10^{-4}
6	−5.76	0.60×10^{-4}
7	−7.72	2.19×10^{-6}
9	−7.67	2.39×10^{-6}

From calculations of ΔG values from ASA studies with STC method, the results obtained indicates that derivatives **1** and **2** appear to have the best binding free energy (see Table 1). Also, the contribution of aminoacids Leu 183, Val 182, Ile 179, Phe 158, Ser140 that show relevant ASA changes is similar in both derivatives and in α-T (Table 2). However it is worth mentioning that in the results obtained from LPC studies in the case of derivative **2** appear some destabilizing contacts (Ser 140, Leu 183 with $-ONO_2$) that are not shown with STC program.

More detailed analyzes of the interactions between protein and ligands calculated with LPC program[28] showed that the hydrogen-bond network and van der Waals contacts are maintained with Phe133, Ser140, Phe158, Ile179, Val182 and Leu183 (as examples, final complex of α-TTP with **1**, **2**, **7** and **9** are shown in Fig. 2). Table 3 gathers the residues involved in the binding site resulting from ligand-receptor α-TTP complexes studied with the molecules **1**, **2**, **7** and **9** in comparison to α-T.

The type of interaction of α-TTP residues varied from one derivative to another (Table 3). Derivative **1** with a natural phytyl chain, showed identical hydrophobic interactions as α-T, involving the methyl group in 2-position interacting with Ile179 and Val182, 5-position interacting with Leu183, 7-position interacting with Ser140, 8-position interacting with Phe133 and Val182, and phytyl side chain interacting with Phe158. In addition derivative **1** shows a hydrogen bond between the oxygen in the hydroxyl group and the OG atom of Ser140. Regarding derivative **2**, hydrophobic interactions are the same as derivative **1**, however there are some destabilizing contacts (hydrophilic–hydrophobic contacts). And the hydrogen bond has disappeared. The mentioned hydrogen bond interaction is also observed for derivatives **7** (distance to the residue: 3.0 Å) and **9** (distance to the residue: 3.1 Å). In addition, in the case of derivatives **7** and **9** there is an aromatic π–π interaction between Phe158 and the phenyl group of SO_2 moiety (distance between centroids of phenyl groups of 4.13 Å and 4.07 Å, respectively). The same moiety in these derivatives also interacts, via a hydrogen bond through one oxygen of the SO_2 group, with Tyr100 hydroxyl group (Table 3).

2.3. Binding to human tocopherol transfer protein

Selected synthesized hybrid compounds (**2–4**, and **7–10**, Chart 1) that showed wide-ranging results in docking studies were tested for their affinity to α-TTP. To determine whether these compounds are ligands for α-TTP we used our previously described methodology[29,30] in which each derivative was studied for its ability to displace the fluorescent tocopherol probe (NBD-Toc, **11**, Chart 1)[31] from the recombinant hTTP as compared to α-T. Analyzing the results, it was observed that some of the studied hybrid derivatives were capable of forming aggregates or micelles, in the assay milieu, when the concentration approached its critical micelle concentration (CMC), consequently this physicochemical parameter was determined for selected compounds (see Section 4 and Supplementary data).[32]

Table 2
ΔG_{bind} (kcal/mol) contribution of those residues that show relevant ASA changes **1**

Residue	α-T	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**9**
Leu 183	−0.11	−0.12	−0.11	−0.04	−0.03	−0.04	−0.03	−0.04	−0.09
Val 182	−0.21	-0.21	−0.27	−0.21	−0.24	−0.21	−0.24	−0.37	−0.37
Ile 179	−0.21	−0.22	−0.22	−0.10	−0.11	−0.10	−0.10	−0.19	−0.19
Phe 158	−0.31	−0.30	−0.31	−0.14	−0.17	−0.14	−0.15	−0.32	−0.32
Ser140	−0.03	−0.10	−0.14	-0.10	−0.13	−0.10	−0.13	−0.12	−0.12
Ser 136	–	–	–	–	–	–	–	−0.08	−0.10

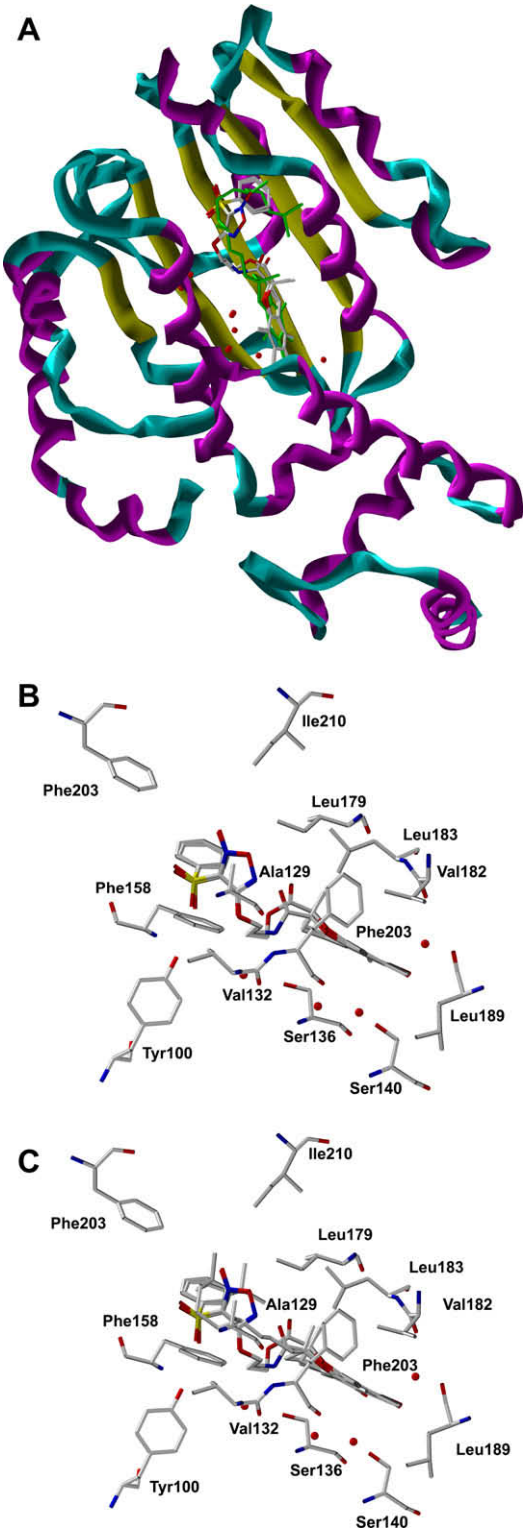

Figure 2. (A) Overview of the complex α-TTP-derivatives **7**, **9** and α-T. Derivative structures are depicted as stick model and the protein is depicted as ribbon. (B) Close-up view of the derivative-binding pocket, derivative structures (**7** and **9**) and aminoacidic residues within the binding pocket are depicted as stick model in gray colour, internal water molecules as red balls, residues within the binding pocket in orange. (C) Close-up view of structural superposition of the derivatives **7**, **9** and α-T. Derivatives **7** and **9** are depicted as stick model in gray colour and α-T in green colour, internal water molecules as red balls, residues within the binding pocket in orange.

As shown in Figure 3A, using derivative **10** as an example (for complete data see Supplementary data), in the presence of the protein-fluorophore mixture with the 6-acetyl derivatives **2**, **4**, **8** and **10**, aggregates form as evidenced by a drastic increase in fluorescence. This kind of behavior was previously observed with cholesterol in the validation studies of NBD-Toc as α-TTP-fluorescent probe.[29] However, the free phenol analogues, **3**, **7** and **9** (shown as example in Fig. 3B, for complete data see Supplementary data) behaved in a more similar way to α-T and showed a comparatively better competition curve than that of the previous compounds. Compound **3**, the free phenol derivative of **4**, competes for 50% of the initial fluorescence at about 15 μM, whereas α-T requires between 1 and 2 μM. Derivative **7**, the free phenol derivative of **8**, competes at low concentrations (initial fluorescence loss) but then a steady increase in fluorescence is observed. The assay was visibly cloudy at concentrations greater than 5 μM clearly indicating that it was not possible to supply soluble material and maintain a true equilibrium condition. From the observed competition below ~5 μM concentration derivative **7** appears to be nearly equal to α-T in its ability to displace NBD-Toc from α-TTP (Fig. S4, Supplementary data). Compound **9**, the free phenol derivative of **10**, is the best behaving one in the group at the complete range of studied concentrations (1–40 μM). It showed improved binding characteristics despite also producing cloudy assay solutions at 10–15 μM. Nonetheless, it clearly reduces the initial fluorescence to nearly the same degree as α-T (~30%).

Despite derivatives **9** and **10** having the same CMC (6 μM), and the lowest CMC values in all the studied compounds (see Supplementary data), the behavior in the α-TTP assay is very different, thus a different factor is playing a role in the failure of derivative **10** in the α-TTP-assay. One could hypothesize, using the docking data, the extra interaction that phenol-derivatives establish with the protein, via Ser140 (see above), promotes the equilibrium displacement according to phenol-milieu to phenol-α-TTP, consequently, they interact better with the protein (also compare results between **3** and **4**, and **7** and **8**, Supplementary data).

Comparing the above results to the calculated ΔG_{bind} values (Table 1) there is some agreement in rank order. Derivatives **7** and **9** classified as the best ligands in binding assays showed good binding energies in docking studies. These derivatives decreased the fluorescence of α-TTP/NBD-Toc to a half of the original fluorescence intensity at 2 μM and 6 μM, respectively, comparable to α-T which required about 1–2 μM to reach the same point. On the contrary, the ΔG_{bind} for compound **2** calculated with STC program (Table 1) is the highest value pointing out a strong interaction, however if we observe the interactions (Table 3) it is possible to notice that the HB does not exist and in addition there are several destabilizing interactions according to LPC program. Therefore the calculated ΔG_{bind} for compound **2** is overestimated in relation to compound **1**.

Therefore, it is possible to hypothesize that the lack of free OH group does not allow this compound to interact adequately with the protein which thus lowers NBD-Toc displacement with the concomitant decrease of fluorescence. We tried to develop the free phenol analog, derivative **1**, but in our hands none of our attempts to synthesize it have been successful.

In any case these results reinforce that a ligand of α-TTP must have in its structure the chroman ring with the free phenol and a side chain capable to interact with Phe158. Moreover, it should be noted that all derivatives were assayed as a mixture of stereoisomers. For compounds **3**, **4**, and **7**–**10**, the *S*-stereoisomers at C-2 corresponds to the same configuration in *R*,*R*,*R*-α-T and NBD-Toc (used in the assays as pure 2*R*-enantiomer). If 2*S*-isomer in compound **2** or 2*R*-isomers in compounds **3**, **4**, and **7**–**10** are as poor ligands as *S*,*R*,*R*-α-T (22 times less active than 2*R*-α-T),[30] then the other isomer (2*R*-isomer or 2*S*-isomers, respectively) interact even better than it can be seen herein, since only half of the sample is effectively competing with NBD-Toc for the active site.

Table 3
ΔG_{bind} and K_d of ligands

Complex/residue	α-T[a]	1	2	7	9
Tyr 100(OH)	—[b]	—	—	HB[c] (O═S═O, 3.1)	HB (O═S═O, 3.1)
Ser 140 (OG)	Ph[d]	HB (O6, 2.9)	Ph/Hy-Ph[e]	HB (O6, 3.0)	HB (O6, 3.1)
Phe 158	Ph	Ph	Ph	π–π (SO_2Ph)	π–π (SO_2Ph)
Ile 179	Ph	Ph	Ph	Ph	Ph
Val 182	Ph	Ph	Ph	Ph	Ph
Leu 183	Ph	Ph	Ph/Hy-Ph	Ph	Ph

[a] OIP according to PDB code.
[b] —: No interaction.
[c] HB: hydrogen bond. Between brackets are the corresponding atoms involved in the interaction together with the distances to the residues in Å.
[d] Ph: aromatic interactions.
[e] Hy-Ph: Hydrophobic interactions. Destabilizing contacts.

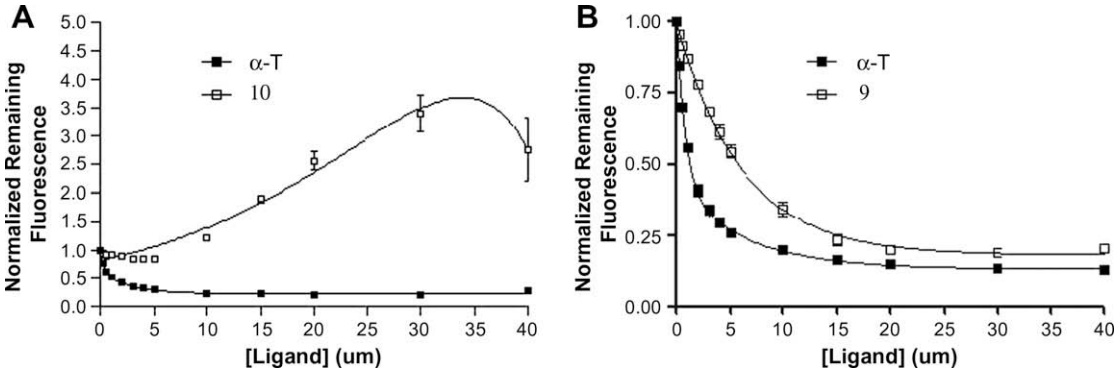

Figure 3. (A) Competition of α-TTP bound NBD-Toc with α-T (■) and **10** (□). (B) Competition of hTTP bound NBD-Toc with α-T (■) and **9** (□). The assay was run in triplicate as follows: Recombinant α-TTP (0.2 μM) was incubated with 1.0 μM of NBD-Toc in SET buffer (250 mM sucrose, 1 mM EDTA, 50 mM Tris, pH 7.37) containing 100 μM of Triton X-100 for 25 min at 20 °C. The fluorescence of NBD-Toc, when bound to hTTP, is exponentially greater than when NBD-Toc is in solution. Once equilibrium has been achieved between the α-TTP and NBD-Toc, small aliquots of competitor ligand or α-T (situated in EtOH stock solutions), reaching different final concentrations, were added. After equilibrium had been reestablished, the changes in fluorescence were recorded.

3. Conclusions

From docking studies we can conclude that derivatives **1**, **2**, **7** and **9** have energy of interaction values similar to α-T. However, experimental affinity of the synthesized compounds to α-TTP demonstrated that furoxanyl-derivatives **7** and **9** are the best binding compounds. The binding efficacy of **7** and **9** is of the same order than that of α-T. This fact suggests that an in vivo transport of these furoxanyl-derivatives could be guaranteed through this transfer protein. In addition, these findings confirm that docking technique will be a very helpful tool to the design novel compounds. Taken together these results and previous one,[8–11] give emphasis to these novel ·NO donors as potential antiatherogenic agents. In this sense, in vivo bioavailability and antiatherogenic properties of these derivatives are in progress.

In order to obtain a direct measurement of the interactions between our hybrid compounds and α-TTP protein other experiments should be performed, that is, saturation transfer difference-NMR experiments, isothermal titration, and/or X-ray studies. These studies will be performed.

4. Experimental

4.1. Docking studies

All calculations were performed using the SYBYL 7.2 program suite [Tripos Inc., St. Louis. Mo 1]. The docking studies were achieved using the FLEXIDOCK module of the SYBYL 7.2 suite of programs.[25]

The structure of the complex hepatic α-TTP/α-T obtained from X-ray (pdb code: 1oip) was edited, protein hydrogen atoms were added and partial charges were calculated using AMBER procedure. Positions of the hydrogen atoms were refined with the use of AMBER force field.

All ligands docked into the active site were created using the Sketch Molecule module of SYBYL 7.2 from vitamin E structure. In all cases, MMFF94 force field and charges were used for geometry optimization of the ligands using conjugate gradient method until the gradient reached 0.05 kcal mol^{-1} $Å^{-1}$. The compounds were manually positioned within the binding pocket of the enzyme by structural alignment with vitamin E in the complex 1oip. Subsequent energy minimization of the initial complexes was performed using MMFF94 force field with geometry optimization of the ligands and the side chains of the enzyme. Energy minimizations were carried out using the Conjugate Gradient procedure until a gradient deviation of 0.01 kcal mol^{-1} $Å^{-1}$ was attained. A distance-dependent dielectric constant was used in all the calculations. These initial complexes were the input structure for docking studies using FLEXIDOCK command. FLEXIDOCK analyzes all possible ligand conformations within the active site and it takes into account both the receptor side chains and the ligand conformations; in other words, it allows a flexible docking to be performed both in the ligand and the receptor. FLEXIDOCK uses genetic algorithms (GAs) which is a very quick method to generate conformations. Judson and Haydon[33] among other authors have shown that GAs provide a good, if not better, performance than other methods of conformational search. During the docking analysis, the protein was considered rigid except the residues involved in the binding site (5 Å) and the ligands were considered flexible. Several runs of FLEXIDOCK were performed using the default FLEXIDOCK parameters, with iterations set to 500–1000 generations per number of rotatable bonds plus six. The final solutions were analyzed and clustered yielding different families. The representative conformer

from each group or family was re-optimized using the above mentioned conditions.

Analysis of the enzyme/ligand complex models generated after docking was based on the distances from the binding site, the hydrogen bond interactions, aromatic and hydrophobic interactions calculated with LPC program and energy of binding and the difference accessible surface area (dASA) obtained with Structural Thermodynamics Calculations V. 4.3(STC). STC performs free energy calculations from the structure of the complex from the change in accessible surface area. From the change in ASA of the different side-chains involved in the binding, the conformational entropy gained for the ligand and the enzyme is calculated. Finally, the total entropy change is calculated as the sum of all the entropic contributions. ΔG_{bind} is the free energy of binding. A negative value for ΔG_{bind} indicates binding is favorable, a positive value for ΔG_{bind} indicates binding in not favorable.

4.2. Tocopherol transfer protein (TTP) binding assays

Recombinant human α-TTP[34] (0.2 μM) was incubated with 1.0 μM NBD-Toc in SET buffer (250 mM sucrose, 1 mM EDTA, 50 mM Tris, pH 7.37) containing 100 μM Triton X-100 at ~20 °C. The fluorescence of NBD-Toc is greatly enhanced on binding to the protein. After the signal reached equilibrium, small aliquots of competitor or α-T dissolved in EtOH (final concentrations between 1 and 40 μM) were added, and the signal was monitored until the observed fluorescence decrease came to a steady value. The final concentration of EtOH did not exceed 1% (v/v).

4.3. Critical micelle concentration determination

CMC of selected derivatives were determined following procedures previously reported (see Supplementary data).[31]

Acknowledgments

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Supplementary data

Supplementary data (additional experimental details: critical micelle concentration determination and results for all compounds and results of the competition of α-TTP bound NBD-Toc with α-T and the rest of the studied compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.10.046.

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