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ARTÍCULO

Prediction of ligand binding energy and anti-inflammatory effect of flavonoids in the glucocorticoid receptor by molecular dynamics simulations and linear interaction energy method

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ABSTRACT

Flavonoids are compounds composed of a three-ring structure with various substitutions formed in plants, from aromatic amino acids phenylalanine and tyrosine, to participate in the light-depend phase of photosynthesis catalyzing electron transport. Their anti-inflammatory properties are of special interest as adverse reactions in humans appear to be rare. For this reason the aim of this work is to try to explain the binding mode of flavonoids and to estimate the binding energy of the compounds to glucocorticoids receptor using the LIE method. Fifteen flavonoids were used to correlate calculated binding energy with experimental values. The squared correlation coefficient achieved is significant (R^2 = 0.74 and p = 0.000) and good description of the binding mode was obtained. Three hydrogen binding zones were identified corresponding to aminoacids Arg81-Gln40, Thr205-Gln111 and Leu33-Asn34. The interaction with the first zone was found related with lower values of energy of binding with statistically significance of 95%.

Keywords: Molecular dynamics; Docking; Glucocorticoids Receptor; Flavonoids.

RESUMEN

Predicción de la energía de unión y efecto antiinflamatorio de flavonoides sobre el receptor de glucocorticoides mediante simulaciones de dinámica molecular y el método linear de energía de interacción

Los flavonoides son compuestos formados por tres anillos con varias sustituciones sintetizados en vegetales de los aminoácidos aromáticos fenilalanina y tirosina para participar en la fase luz-dependiente de la fotosíntesis transportando electrones. Sus propiedades antiinflamatorias son de especial interés debido a que las reacciones adversas que estos producen en humanos son raras. Por esta razón, el objetivo del presente trabajo es explicar el modo de unión de estas moléculas y estimar la energía de unión al receptor de glucocorticoides utilizando el método LIE. Se utilizaron 15 flavonoides para correlacionar sus energías de unión estimadas con las experimentales. El coeficiente de correlación alcanzado fue significativo (R^2 = 0,74 y p = 0,000) y se obtuvo una buena descripción del modelo. Se encontraron tres zonas de unión por puente de hidrógeno correspondiendo a los aminoácidos Arg81-Gln40, Thr205-Gln111 and Leu33-Asn34. La primera zona se pudo correlacionar con menores valores de energía con una confianza del 95%.

Palabras clave: Dinámica molecular; Docking; Receptor glucocorticoides; Flavonoides.

1. INTRODUCTION

Flavonoids are compounds of low molecular weight, derived from the secondary metabolism of a wide range of plants. Flavonoids are composed of a three-ring structure with various substitutions and are formed in plants, from aromatic amino acids phenylalanine and tyrosine, to participate in the light-depend phase of photosynthesis catalyzing electron transport. In plants, they are usually found

as glycosylated or sulfated derivatives and only as phenolic acid derivatives after biotransformation in the intestine conducted by microorganism. Their antioxidant, anti-inflammatory and cytoprotective activities are described extensively in literature and are known since ancient times. Anti-inflammatory properties are of special interest as adverse reactions in humans appear to be rare, even with a daily exposure to flavonoids-containing food.

Several mechanisms are suggested for anti-inflammatory effects of flavonoids. One of the most proved and complex mechanisms, implicates an inhibition in the release of inflammatory molecules such histamine, tryptase, IL-6, IL-8 and TNF-alfa, from activated mast cells trough a phosphorylation of MACEDONIA (Mast Cell Degranulation Inhibitor Agent) protein, involved in exocytosis (1). The underlying mechanism of this action is based on an activation of protein kinase C (PKC) calcium-independent and an inhibition of other PKC enzymes and tyrosin-kinases that play a major role in the inflammatory response.

Another mechanism proposed to justify the anti-inflammatory activity, suggests an inhibition of enzymes of the cyclooxygenase (COX) family, which produces the transformation of araquidonic acid into proinflammatory prostaglandins, competitively (2) or decreasing its transcription (3).

However, in a recent work, Nishizaki *et al.* (4) reported that flavonoids are powerful agonist of glucocorticoid receptors. Glucocorticoids bind the nuclear receptor, accelerating the transcription of genes that codify for proteins like lipocortin-1 which strongly inhibits the effect of phospholipase-2 that provides araquidonic acid which is the substrate of COX. Thus, an indirect anti-inflammatory effect can be expected by the interaction of flavonoids with the glucocorticoid receptor (GR).

In the light of the diversity of plants with anti-inflammatory effect mediated by flavonoids (5), it is of great value to develop a method to predict the binding affinity to GR. For this reason the aim of this work is to try to explain the binding mode of flavonoids and to estimate the binding energy of the compounds based on experimental values from gene expression assays and the linear interaction energy (LIE) approximation (6).

2. MATERIALS AND METHODS

2.1. Ligands/Enzyme models

To achieve a reliable model, the structures of the ligands (Table 1) were energy minimized, in first place using the MM2 force field, and afterwards with the semi-empirical Hamiltonian AM1 (Austin Model 1), as implemented in MOPAC 7.1. The structures resulting from this procedure were used as the starting point of docking assays.

The atomic model of GR from X-ray diffraction studies was obtained from Brookhaven Protein Data Bank (PDB code: 1M2Z). Following, the ligand binding domain (residues from 521 to 777) was extracted discarding all crystallographic water and the ligand (dexamethasone). To avoid the high energy interactions present in the crystal structure, a two parts energy minimization protocol was carried out. In the first part, the structure was minimized by 400 steps of steepest descents (SD), carefully observing the root mean square deviation (RMSD) from the initial crystallographic positions to avoid distortion of the structure. In the last part, 1500 steps of Polak-Ribiere conjugate gradient (GC) were applied with the same considerations about distortion of the structure. This protocol was carried out using the GROMOS 96 43a1 force field and the suite GROMACS 4.0.3.

2.2. Docking

All docking studies were carried out by the program Autodock4 (7) version 4.0.3 which allows a very fast energy evaluation using precomputed grids of affinity potentials for rigid docking. In order to explore the conformational space of the ligands, all torsional bonds in substrates were set free to perform flexible docking while the enzyme was kept rigid. Polar hydrogens and Gasteiger charges were assigned by the respective modules in Autodock Tools (7).

With every ligand, we developed a rigid docking assay with a grid box of 40 amstrong × 40 amstrong × 40 amstrong placed at the crystallographic coordinates of the ligand with a spacing of 0.375 amstrong between points, assuring coverage over the active center.

All the grid maps used to represent the protein in the rigid docking were calculated by AutoGrid.

Finally, the empirical free energy function and the Lamarckian genetic algorithm were used, applying a standard protocol with an initial population of 150 randomly placed individuals, a maximum number of 2.4×10^7 energy evaluations, a mutation rate of 0.02, a crossover rate of 0.80, and an elitism value of 1.

The results were clustered according to a RMSD criterion and were classified taking the predicted energy of binding into account. In all cases the most favorable conformations were selected according to the following criteria: best energy and best superimposition with the crystallographic ligand. Suitable conformations were used in molecular dynamic studies for assessing the stability and the energy of the complex.

2.3. Molecular dynamics

The complexes ligand-enzyme were evaluated in molecular dynamics simulation. All the topological parameters for the enzyme were created by GROMACS programs and the parameters of ligands were built by the Dundee PRODRG Beta Server (8). The complexes were solvated in a box of SPC/E water, neutralized with sodium ions and then energy minimized by a two-steps protocol as described before for the enzyme.

Following the minimization, a simulation of 300 ps at 298K and 1 atm with pressure coupling using Parrinello-Rahman method (9) was performed using the leapfrog algorithm (isobaric-isotherm, NPT assemble and periodic boundary conditions) with constraints in all bonds using LINCS algorithm (10). Particle-Mesh-Ewald (PME) summation was applied dealing with long-range electrostatics (11) and a 10 amstrong cut-off for van der Waals interactions was used. Energy and coordinates were recorded each picosecond to estimate the energy contribution and the possible interactions between ligand and the protein.

2.4. Linear interaction energy

The LIE method is based on the assumption that ΔG of the system depends linearly on changes in the van der Waals and electrostatic components. This is supported by observations that the free energy of solvation on non-polar moieties often scale linearly with respect to variables characterizing the size of the solute (12).

To perform the binding energy estimation for every complex, we used this approach. LIE method evaluates separately the electrostatic and van der Waals interaction energies of the ligand in bound and free states. For this purpose, we carried out new molecular dynamics simulations of all ligands in water to measure the coulombic and van der Waals contributions of the free ligands with identical protocol and considerations to the complex dynamics.

The approximated binding energy is obtained as it is shown:

$$\Delta G_{bind} = \alpha(<E^{vdw}>_{complex} - <E^{vdw}>_{free}) + \beta(<E^{qq}>_{complex} - <E^{qq}>_{free})$$

where $<E^{vdw}>_{complex}$ and $<E^{vdw}>_{free}$ denote the average van der Waals interaction energies in the bound and free forms, and $<E^{qq}>_{complex}$ and $<E^{qq}>_{free}$ denote the average electrostatic interaction energies in the bound and free forms. The value of α, strongly depends on the system, the force field and the computational methods applied. For this reason, a proper value should be determined by comparing the experimental and calculated binding energies. In addition, β value was originally fixed to 0.5, however the study of solvation energies of various small substrates showed that β decreases with the number polar groups like hydroxyl so a consideration to this point have to be done.

Training set to determine the values of α and β, was composed with dexamethasone, betamethasone and cortisol, all well known GR ligands with Kd (constant of dissociation) experimentally measured (Table 2). Exactly the same protocol for flavonoids was followed for the three training compounds and 300 ps bound and free simulations were carried out for each ligand.

To obtain binding energy values for the compounds in the training set, we applied the equation:

$$\Delta G_{bind} = -RT \ln Kd$$

where R is the gas constant, T is temperature (Kelvin) and Kd is the dissociation constant. The calculated ΔG_{bind} values were obtained by the LIE approximation using the energy contributions retrieved from MD trajectories of free and bound ligand and were used in a linear regression test to determinate if they are statistically related with the experimental ΔG_{bind} using the GNU/PSPP 0.6.1 program (13).

The use of PME (Particle-Mesh-Ewald) dealing with the long range electrostatic interactions was specially considered as in LIE method only short-range contributions are taking into account. The results of the test yield in a very low energy contribution of the PME to the total electrostatic energy so all effects of periodic boundary conditions and PME can be neglected for the low size of the system.

3. RESULTS

3.1. Docking

In a first approach, dexamethasone was evaluated in a docking assay to determine if the protocol was able to predict the crystallographic position with an acceptable value of RMSD and to validate the method for further evaluation of flavonoids. The docked conformation with the best energy was found with a RMSD value less than 1.0 amstrong, so the method was confirmed to be capable of found the correct conformation. Identical experiments were carried out with the rest of ligands in the training set (betamethasone and cortisol) and similar results were obtained, and therefore a binding mode for molecule in training set was identified.

Once the method was validated, we applied it to the compounds of the test set (Table 1). The results of these experiments were evaluated and were used to identify potential interactions with the GR. Mainly two kinds of interactions were found in the training and test sets: Hydrogen bonds and hydrophobic interactions. The first kind is due to the interactions that occur between the hydroxyl and ketone groups of the ligands and polar residues surrounding the cavity that binds the ligand. The second kind occurs when aromatic rings of the ligands interact with highly apolar residues around the cavity.

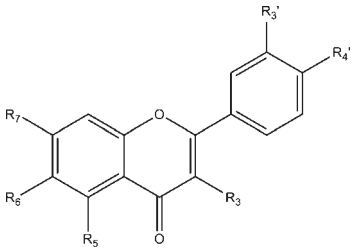
Analyzing the docked conformations, we identified three zones where hydrogen bonds can be found. The first one corresponds to the residues of Arg81 and Gln40 where ketone groups of the ligands tend to interact, the second one is placed near residues Gln111 and Thr205 and bind with hydroxyacethyl chains. In the last one, Leu33 and Asn34 interact with hydroxyl group in the C ring of training set molecules (Figure 1 and 2). Hydrophobic interactions in the pocket are due to Met30, Phe31, Leu33, Val41, Trp70, Met71, Met74, Met115, Tyr210, Leu219 and Ile 22 that surround the ligand and form the cavity in which this binds. For the test set, the distance and nature of the interactions are summarized in the Table 2. Apart from the residues identified in the training set, new binding modes were found that implies new hydrogen bonds with Cys202, Leu198 and Met30 backbones.

3.2. Molecular dynamics

MD trajectories were inspected to check the stability of complexes, using de RMSD values of Cα atoms compared to the initial conformation. After 60 ps of the production run, the complexes were found stable in all cases, with values of RMSD ranging from 1.29A ± 0.21 to 1.58A ± 0.22 for the whole simulation. Despite of this short time needed to stabilize complexes, first 100 ps of the MD run were discarded as equilibration time in order to achieve even higher levels of confidence in the position of the ligands and the aminoacids of the binding pocket.

Hydrogen bonding during MD simulation between ligand and the surrounding environment were analyzed with g_hbond (GROMACS Hydrogen bond analysis tool) to check the stability in time of the docking results. In addition, energy values of complexes and free ligand forms were extracted and considered for LIE analysis.

Table 1. **Flavonoids included in the test set**



No	Compound	R3	R5	R6	R7	R3'	R4'	EC150*
1	Flavone	H	H	H	H	H	H	26.1
2	5-hydroxyflavone	H	OH	H	H	H	H	6.0
3	5-methoxyflavone	H	OCH3	H	H	H	H	5.5
4	6-hydroxyflavone	H	H	OH	H	H	H	7.1
5	6-methylflavone	H	H	CH3	H	H	H	3.6
6	6-methoxyflavone	H	H	OCH3	H	H	H	0.7
7	6-Chloroflavone	H	H	Cl	H	H	H	12.6
8	7-Hydroxyflavone	H	H	H	OH	H	H	3.1
9	7-methoxyflavone	H	H	H	OCH3	H	H	21.9
10	3-hydroxy-6-methoxyflavone	OH	H	OCH3	H	H	H	10.3
11	5,7 dihydroxyflavone	H	OH	H	OH	H	H	1.5
12	5,6,7 trihydroxyflavone	H	OH	OH	OH	H	H	18.9
13	4',5,7 trihydroxyflavone	H	OH	H	OH	H	OH	5.2
14	5,7 dihydroxy-4'-methoxyflavone	H	OH	H	OH	H	OCH3	2.1
15	3',4',5,7-tetrahydroxyflavone	H	OH	H	OH	OH	OH	18.0

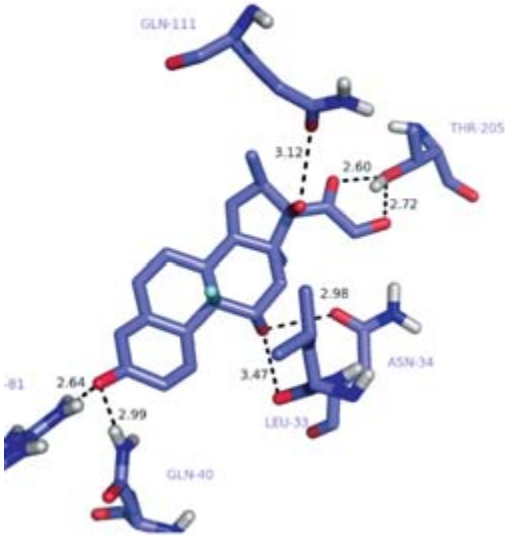


Figure 1. Dexamethasone-GR complex. Binding mode of dexamethasone is shown with hydrogen bonds.

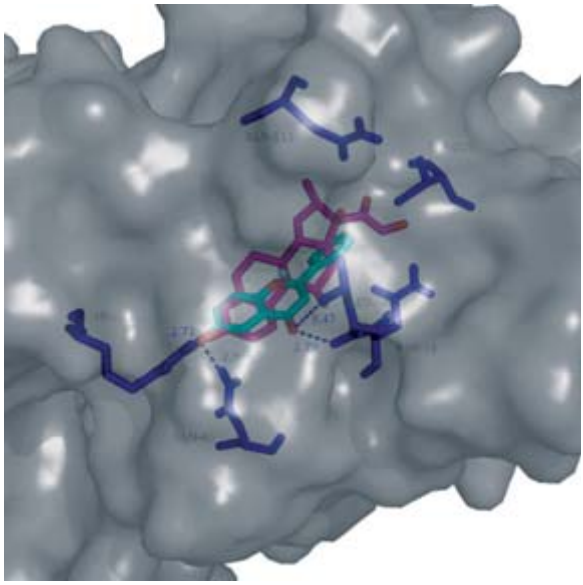


Figure 2. 6-methoxyflavone and dexamethasone. Flavonoid, in blue, superimposed on dexamethasone, in pink.

Table 2. **GR-flavonoids interactions.**

Compound	LEU33	ASN34	GLN40	ARG81	GLN111	THR205	Others
1	—	—	—	—	—	2.2	—
2	—	2.8	—	—	—	1.9	2.7-3
3	—	2.9-3.5	—	—	—	3.0	3.3
4	—	—	3.2	3.0	—	—	—
5	—	—	—	—	—	—	—
6	2.8	—	3.0	2.7	—	—	—
7	—	—	—	—	—	—	—
8	—	2.9-3.0	—	—	—	—	2.8-2.6-3.3
9	—	—	—	—	—	2.7	—
10	—	—	—	—	—	3.0	—
11	—	—	—	—	—	2.9	3.0-2.7
12	—	—	—	—	3.1	2.5	2.9-3.1
13	—	2.4-2.9	3.2	3.0	—	—	3.1-2.8
14	—	2.7	3.0	3.2	—	3.0	2,7-3.1-3.1
15	2.9	2.7	3.0	3.2	—	3.0	3.1-3.1-2.7
Dexamethasone	3.5	3.0	3.0	2.6	3.1	2.6-2.7	—
Betamethasone	3.3	3.0	2.9	2.7	3.3	2.9-3.0	—
Cortisol	3.0	3.0	3.1	2.8	3.1	2.6-3.1	—

Distances are shown in amstrong. Other summarizes Cys202, Leu198 and Met30 interactions.

Prior to extract energies from flavonoids complexes, we constructed a training set as described before. The values of the calculated and experimental energies are shown in Table 3, and the results for the best regression test can be seen in Figure 3. Best correlation was found for the pair values $\alpha = 0.42$ and $\beta = 0.5$. The value of α for van der Waals contribution is in good agreement with the fact that hydrophobic interactions in the case of GR are of great importance and the value of β is the same in the original method proposed for Aqvist *et al.* (6). However, according to Aqvist *et al.* (12) hydroxyl (OH) groups may affect β, decreasing its value the greater their number, hence a correction for the number of hydroxyl groups in molecules of the test set should be done, as in test set all the

ligand have 3 OH groups. To correct the underestimation of the electrostatic contribution in the molecule with less than 2 OH groups, $\beta = 0.52$ was chosen and to correct the overestimation of the same contribution in the case of more than 4 OH, $\beta = 0.48$ was selected.

After best coefficients were chosen, we used them to calculate the binding energy of the test set, checking obtained values with experimental values of activity in an *in vitro* gene assay (4) expressed as EC150 or the concentration of substance to produce 150% stimulation of luciferase (GR induced expression) activity.

Table 3. **Training set energy values.**

Compound	**Log Kd**	**ΔG_{exp} (KJ/mol)**	**ΔG_{calc} (KJ/mol)**
Dexamethasone	8.47[a]	–48.3	–48.38
Betamethasone	8.45[a]	–48.75	–47.56
Cortisol	8.15[b]	–46.5	–48.96

(a) Values from EMEA (14). (b) Values from Eliard *et al*. (15).

As can be seen in Table 4, higher electrostatic contributions are found when more than 2 OH groups are present in the molecule. Indeed, this effect is due to an overestimation of the force field of the electrostatic interactions because the polarization effects are not

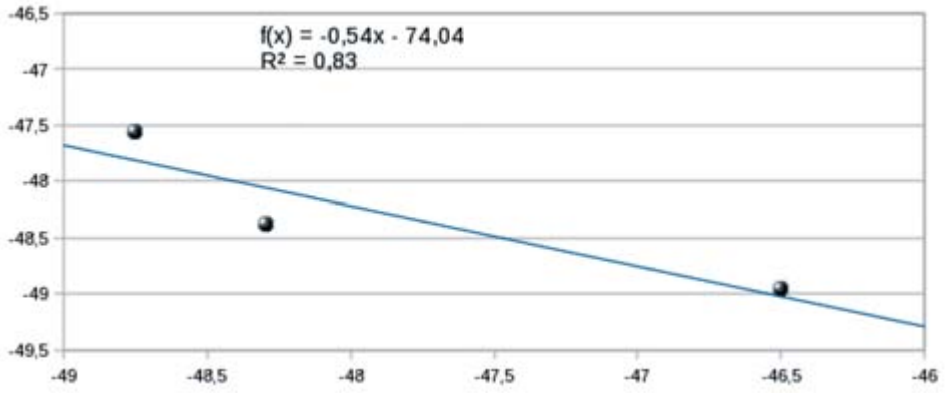


Figure 3. Training set regression test of calculated and experimental binding energies.

properly described, so lower values of b should be required in some cases.

Table 4. **Test set energies and LIE parameters.**

Compound	Free Evdw (KJ/mol)	Free Eqq (KJ/mol)	A	B	OH Groups	ΔG_{calc} (kcal/mol)
1	12.727	78.981	0.42	0.52	0	–10.73
2	12.817	79.792	0.42	0.52	1	–11.21
3	12.830	79.787	0.42	0.52	0	–11.21
4	13.328	82.810	0.42	0.52	1	–11.64
5	13.147	81.671	0.42	0.52	0	–11.48
6	13.586	84.418	0.42	0.52	0	–11.87
7	13.327	82.770	0.42	0.52	0	–11.24
8	12.874	79.895	0.42	0.52	1	–11.23
9	12.710	78.919	0.42	0.52	0	–10.72
10	13.599	84.461	0.42	0.52	1	–11.47
11	12.846	79.876	0.42	0.50	2	–11.41
12	13.299	82.791	0.42	0.50	3	–10.84
13	13.762	85.701	0.42	0.50	3	–11.63
14	13.683	85.101	0.42	0.50	2	–11.55
15	13.686	85.188	0.42	0.48	4	–10.75
Dexamethasone	13.805	85.706	0.42	0.50	3	–11,57
Betamethasone	13.578	84.266	0.42	0.50	3	–11.38
Cortisol	13.874	86.480	0.42	0.50	3	–11.71

Figure 4 shows the regression test performed between the values of calculated energy of binding and the experimental values of EC150. The squared correlation coefficient was found significant (R^2 = 0.74 and p = 0.000), hence a good correlation between calculated energy values and the agonist effect of the compounds can be expected.

However, although an acceptable value of correlation was found, in line with similar recent studies (16), some authors found slight

precision enhancements taking all possible orientations of ligands in the binding pocket into account (17), at the cost of reducing the time of the molecular dynamic simulations and obtaining less confidence in the stability of complexes. Thus, in this work, we chose improved security in the stability of complexes to an increase of conformations to be considered while maintaining computational costs affordable.

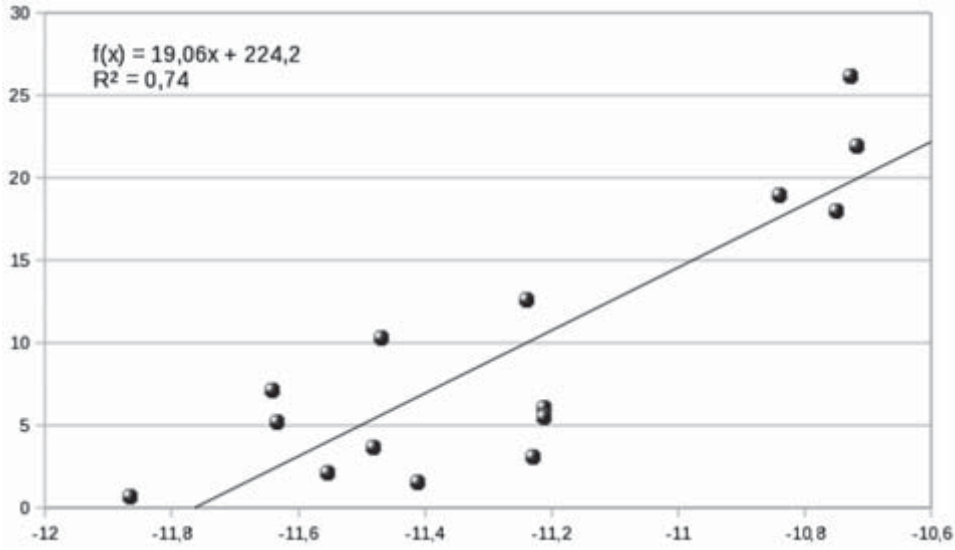


Figure 4. Correlation between calculated binding energy and EC150 for the test set.

3.3. Binding modes and molecular structure considerations

Evaluated flavonoids have a high shape similarity with natural agonist of GR. Indeed, in almost all cases a good overlap between cortisol and the test set molecules can be observed. In order to extract the most relevant structural features of the ligands, the presence of hydrogen bonds, the shape and the calculated binding energy were considered as the principal components when reviewing its capacity as agonists.

Analyzing the test set, two key factors can be identified: number and disposition of OH groups. Figure 5 shows the dependence between the number of OH groups and the energy of binding. In

fact, between 2 and 3 hydroxyl groups the best average energy of binding can be found, due to a better disposition for hydrogen bonding interactions with cavity residues.

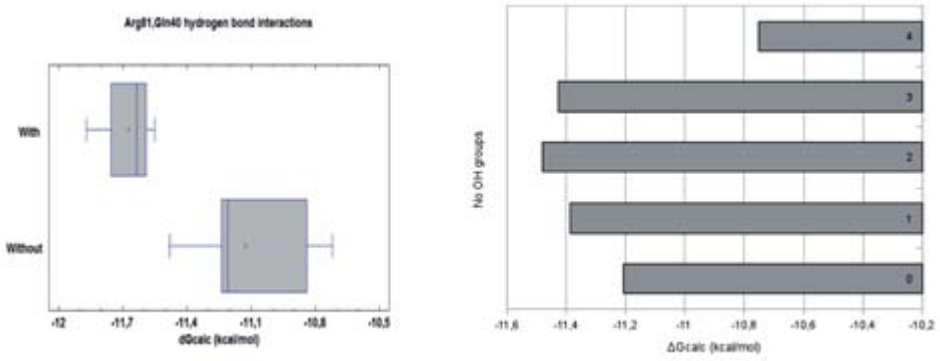


Figure 5. Statistical analysis of Arg81 interaction and energy dependency of number of OH groups.

Regarding the spatial arrangement of the groups, the analysis of the values in Table 2 clearly shows a trend towards better energy when introducing interactions that mimic those produced by the ligands in the training set, especially with the residues Arg81 and Gln40 and with Thr205. A statistical analysis of the two samples (with and without interaction at Arg81-Gln40) was carried out to identify the relevance of the interaction (t-value). The difference in average energy values was found within a significance of 95% ($p = 0.0049$). The rest of interactions were not found relevant to achieve lower energies of binding.

4. CONCLUSIONS

Here, we developed a computational model to describe the interactions of flavonoids with glucocorticoid receptor in high detail, allowing the design of new anti-inflammatory agents based on the hydroxyflavone skeleton. Hydrogen bonding was found to be the most important type of interaction, in special with residues Arg81 and Gln40 (with statistical significance) and Thr205.

In addition a new method is presented to predict the relative potency of flavonoids as glucocorticoids receptor agonists, with a good correlation with *in vitro* experimental values (R^2 = 0.74 and p = 0.000), and with the advantage of the LIE method, that allows to include the effects of solvent.

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