

Research Article

Computer-aided Design of Coumarin Inhibitors of Quinone Oxidoreductase-1 (NQO1) with a Favorable Pharmacokinetic Profile

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Abstract

Over the last ten years, cancer therapies have struggled with drug resistance. In this report, we explore new coumarin (COU) compounds designed to inhibit the enzyme NQO1, which shows potential for effective treatment due to their favorable predicted drug properties. Three-dimensional (3D) models of NQO1-COU_x complexes were generated through *in situ* modifications of the crystal structure of NQO1-COU12 (PDB entry code: 3JSX), which served as the reference compound for a training set of 22 and a validation set of 6 VCOUs with known experimental inhibitory potencies. To identify the active conformation of COU1-22, we developed a gas-phase quantitative structure-activity relationship (QSAR) model that established a linear correlation between the calculated enthalpy of NQO1-COU complex formation and the values of experimental activities. Subsequently, we screened the Virtual Compound Library (VCL) using Lipinski's Rule of Five and the PH4 model, then assessed the potency of the new COU analogues using the retained QSAR model. The pharmacokinetic profile of the analogues obtained was also evaluated using the linear correlation equation derived from the QSAR model. The coefficient of determination (R^2), the Leave One Out (LOO) cross-validated Squared and the Standard error of regression σ for this equation are 0.91, 0.94 and 0.14, respectively, thus revealing the high predictive power of this model. Similarly, the PH4 model, with a correlation coefficient of 0.91, demonstrated robust predictive power. A comprehensive screening of the COU virtual analogue library yielded a total of 63 drug candidates with oral bioavailability, among which the most promising compounds exhibited a predicted potency of 12.22 and a favorable pharmacokinetic profile. The integration of Quantitative Structure-Activity Relationship (QSAR) techniques and *in silico* screening, based on the PH4 model, has enabled us to propose potent anticancer candidates with optimal pharmacokinetic profiles.

Keywords

Coumarins, NQO1, Drug Resistance, Anticancer Therapy, Virtual Screening, Pharmacokinetics

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1. Introduction

NAD(P)H Quinone Oxidoreductase-1 (NQO1) is an important enzyme involved in cellular redox homeostasis, playing a dual role in both detoxification of quinones and activation of certain anticancer drugs [1]. However, its overexpression in various cancers, including pancreatic and colorectal carcinomas, has made it a potential therapeutic target [2]. Inhibiting NQO1 could disrupt tumor cell metabolism and sensitize them to oxidative stress, paving the way for novel anticancer strategies [3].

Coumarin-based molecules, known for their bioactive properties, have shown promising inhibitory activity against NQO1 [4]. However, the development of selective and potent inhibitors requires a rational design approach to optimize binding affinity, pharmacokinetics, and toxicity profiles [5]. In this context, computer-aided drug design (CADD) plays a crucial role in accelerating the identification and optimization of lead compounds [6].

This study explores the computer-aided design of coumarin-based NQO1 inhibitors, integrating molecular docking, quantitative structure-activity relationship (QSAR) models, and molecular dynamics simulations [7]. *In silico* methods enable the identification of the most promising candidate compounds prior to synthesis, thereby minimizing experimental costs and significantly improving the efficiency of the drug discovery process [8]. Ultimately, this research aims to develop optimized therapeutic candidates with improved selectivity and bioavailability, offering new perspectives for cancer treatment [9].

Structural studies have shown that NQO1 exists as a homodimer, with each subunit containing a molecule of flavin adenine dinucleotide (FAD) that is non-covalently bound to the protein [10]. The FAD is positioned such that its isoalloxazine ring forms the floor of the active site cavity. The two active sites are located at opposite ends of the dimer and contain residues from both monomers [10]. The FAD pocket, a buried cavity, is surrounded by the residues Pro68, Trp105, Phe106, Tyr126, Tyr128 and Phe178. The more exposed access pocket, bounded by Tyr128, Met131, Gly149, Gly150, Met154, His161, His194, Phe232 and Phe236 [4]. The catalytic cycle of NQO1 operates through a "ping-pong" mechanism, which occurs in two distinct steps: the transfer of hydride from NAD(P)H to the FAD cofactor, followed by the release of NAD(P)⁺ and the transfer of hydride from the reduced cofactor to the substrate. Compound 1 is a potent competitive inhibitor of NQO1, competing with NAD(P)H for binding to NQO1, thereby preventing electron transfer to FAD [11]. Compound 16 (old identification) or COU12 is frequently used to study the functional consequences of NQO1 inactivity in cells, and recently, much of the research involving 16 has focused on its ability to suppress the malignant phenotype of pancreatic cancer cells both *in vitro* and *in vivo* [12]. NQO1 expression is upregulated in pancreatic

cancer, and the underlying mechanism by which 16 acts is believed to interfere with NQO1's ability to protect cells against oxidative damage [13]. However, it is known that 16 has a variety of other biochemical effects, such as mitochondrial uncoupling, which may complicate the interpretation of NQO1's cellular properties [14].

Starting with the *in-situ* modification of the crystal structure of the NQO1-COU12 complex (PDB: 3JSX), we developed a quantitative structure-activity relationship (QSAR) model that correlated the Gibbs free energies of NQO1-COUx complex formation with the PIC_{50}^{exp} determined from experimental IC_{50}^{exp} values.

This allowed us to identify the active conformation of COUs at the active site of NQO1 from cancer using a molecular mechanics-Poisson-Boltzmann (MM-PB) complexation approach. Utilizing this active conformation, we created a 3D QSAR pharmacophore for NQO1 inhibition (designated PH4). A large virtual library of compounds sharing the COU scaffold was generated and screened *in silico* against the PH4 pharmacophore. The screening results revealed virtual hits with predicted inhibitory potencies (pIC_{50}^{pre}) larger or (IC_{50}^{pre}) lower than that of the most potent training compound, COU1. Subsequently, these hits underwent complexation simulations to evaluate their predicted inhibitory activities, and their ADMET profiles were calculated for further assessment.

2. Methods

Training and validation sets

The training and validation set for the coumarin analog inhibitors of human NQO1 used in this study were obtained from the literature [4]. The experimental values (IC_{50}^{exp}) exhibit a broad range, spanning from 0.18 to 1221 nM, covering over four orders of magnitude. This wide distribution makes them well-suited for developing a robust QSAR model. Among the 28 compounds considered, 22 were allocated to the training set (TS), while 6 compounds were selected for the validation set (VS).

Model building

Three-dimensional (3D) models of the free inhibitors (I), the free NQO1 enzyme (E), and the enzyme-inhibitor complexes (E) were created based on the high-resolution crystal structure (2.45 Å) of a reference complex with the inhibitor COU12 (PDB code: 3JSX) [4]. These models were generated using the graphical interfaces of the molecular modeling tools Insight-II [15] and Discovery Studio 2.5 [16].

Molecular mechanics

The modeling of the COU and the PL ligand complexes was carried out using molecular mechanics, applying the CFF force field [17], as outlined in previous studies [18]. This

process involved optimizing the geometry of the ligand-enzyme interactions by minimizing energy, allowing for a more accurate representation of the binding and structural characteristics of the complexes.

Conformational research

The conformations of the free inhibitors were derived from their bound conformations within the protein-ligand (PL) complexes. This was realized through the implementation of a stepwise relaxation process, thereby enabling the structures to undergo gradual adjustment until they attained the nearest local energy minimum, in accordance with the procedure delineated in preceding studies [19].

Gibbs Free Energies Solvation

Ligand-receptor interactions take place in a solvent environment, which affects the binding process through hydrogen bonding and solvation effects. To account for these influences, the electrostatic component of the Gibbs free energy (GFE) was calculated by solving the nonlinear Poisson-Boltzmann equation [20], incorporating ionic strength. This computation was performed using the Delphi module implemented in Discovery Studio 2.5 [16], according with the approach detailed in previous studies [19].

Interaction energy

The interaction energy (E_{int}) between the enzyme residues and the inhibitor was determined using the CFF force field, following the methodology previously reported [21].

Generation of pharmacophores

The 3D-QSAR (PH4) pharmacophore generation protocol in Discovery Studio [16], based on the Catalyst HypoGen algorithm [16], was used to develop the PH4 model for NQO1 inhibition, following the approach previously described [19].

ADME properties

The pharmacokinetic profile of COUs was assessed using the QikProp program [22], which predicts key ADME (Absorption, Distribution, Metabolism, and Excretion) properties. This analysis was conducted by the methodology previously described [19].

Virtual library generation

The virtual library was constructed with the protocol previously established, involving the systematic generation and

screening of molecular structures based on predefined chemical criteria and computational modeling techniques [21].

ADME based library

The orientation of the virtual library was carried out based on multiple selection criteria, including molecular alignment, binding interactions, and geometric constraints, in accordance with the approach previously described [19].

Pharmacophore-based library search

The pharmacophore model (PH4), derived from the bound conformations of COUs at the NQO1 active site, was used as a tool for searching the library, as previously described [19].

Inhibitory power prediction

For each group within the targeted library subset, the conformer that exhibited the best alignment with the PH4 pharmacophore was selected for in silico screening using the complexation QSAR model. The $\Delta\Delta G_{com}$ value for each newly selected analog was calculated to estimate its NQO1 inhibitory potency (pIC_{50}^{pre}). This prediction was integrated into the target-specific scoring function, as defined in equation (1), which was parameterized using the COU inhibitor training set within the complexation QSAR model [4].

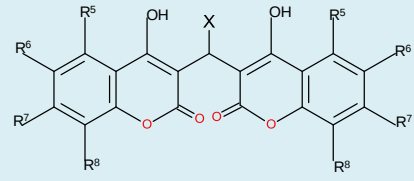
$$pIC_{50}^{exp} = -\log_{10} IC_{50}^{exp} = a \cdot \Delta\Delta G_{com} + b \quad (1)$$

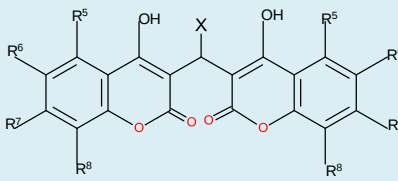
3. Results

Training and validation sets:

A total of 28 COUs (Table 1) were selected from a series of compounds with experimentally determined properties, all originating from the same laboratory [4]. Their measured inhibitory activities ($0.18 \text{ nM} \leq IC_{50}^{exp} \leq 1221 \text{ nM}$) [4] cover a sufficiently broad concentration range to support the development of a robust QSAR model. The ratio between the training and validation set sizes is essential for accurate classification but is limited by the availability of homologous compound datasets in the literature [23]. In this study, a training set of 22 COUs and a validation set of 6 VCOUs were constructed using the appropriate module in Discovery Studio 2.5 [16].

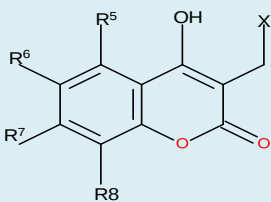
Table 1. NQO1 inhibitors (compounds from 1 to 15) used in the development of the QSAR inhibitor binding model. Group R is numbered in the table column as follows: R5, R6, R7, R8 and group X index [4].

						
Compound identification	R ⁵	R ⁶	R ⁷	R ⁸	X	IC_{50}^{exp} (nM)
1	H	H	H	H	H	2,6

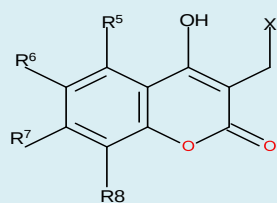


Compound identification	R ⁵	R ⁶	R ⁷	R ⁸	X	IC ₅₀ ^{exp} (nM)
2	H	CH ₃	H	H	H	14,00
3	OCH ₃	H	H	H	H	2,80
4	H	OCH ₃	H	H	H	11,00
5	H	H	OCH ₃	H	H	6,00
6	H	F	H	H	H	4,50
7	H	H	F	H	H	3,80
8	H	Cl	H	H	H	9,00
9	H	Br	H	Br	H	4,80
10	H	CH ₃	CH ₃	H	H	0,41
12	H	H	C ₄ H ₄	C ₄ H ₄	H	0,18
13	H	H	CH ₃	CH ₃	H	0,42
14	H	H	H	H	CO ₂ Et	1221,00
15	H	H	H	H	C ₃ H ₇	588,00

Table 2. NQO1 inhibitors (compounds from 16 to 29) used in the development of the QSAR inhibitor binding model. Group R is numbered in the table column as follows: R⁵, R⁶, R⁷, R⁸ and group X index [4].



Compound identification	R ⁵	R ⁶	R ⁷	R ⁸	X	IC ₅₀ ^{exp} (nM)
16	H	CH ₃	CH ₃	H	1-naphthyl	7,70
17	H	CH ₃	CH ₃	H	2-naphthyl	2,50
18	H	CH ₃	CH ₃	H	phenyl	39,00
19	H	H	C ₄ H ₄	C ₄ H ₄	1-naphthyl	6,30
20	H	H	C ₄ H ₄	C ₄ H ₄	2-naphthyl	2,20
21	H	H	C ₄ H ₄	C ₄ H ₄	phenyl	35,00
22	C ₄ H ₄	C ₄ H ₄	H	H	1-naphthyl	6,30
23	C ₄ H ₄	C ₄ H ₄	H	H	2-naphthyl	6,00
24	H	C ₄ H ₄	H	H	phenyl	15,00
25	H	H	H	H	1-naphthyl	24,00
26	H	H	H	H	2-naphthyl	14,00



Compound identification	R ⁵	R ⁶	R ⁷	R ⁸	X	IC ₅₀ ^{exp} (nM)
27	H	H	H	H	phenyl	144,00
28	H	H	H	H	3,4-dimethyl phenyl	31,00
29	H	CH ₃	CH ₃	H	3,4-dimethyl phenyl	9,90

3.1. QSAR Model

One-descriptor QSAR model: Each of the 22-training set (TS) and 6 validations set (VS) complexes was generated through in situ modification of the crystal structure of the refined NQO1 complex (PDB code: 3JSX) [4], following the procedure detailed in the Methods section. The relative Gibbs free energy (GFE) of NQO1 complex formation ($\Delta\Delta G_{com}$) was computed for each of the 28 optimized enzyme-inhibitor complexes. Table 3 shows the calculated values ($\Delta\Delta G_{com}$) for

the molecules used for the training set (TS) [4]. Table 4 shows the molecules used in the validation set (VS) [4].

To establish a quantitative structure-activity relationship (QSAR), the experimental inhibitory activities (IC_{50}^{exp}) of COUs were converted to (pIC_{50}^{exp}), then correlated with the computed $\Delta\Delta G_{com}$ using linear regression (Table 3). The resulting strong correlation confirmed the active bound conformation of COUs within the NQO1 binding site, leading to the definition of the NQO1 inhibition pharmacophore (PH4).

Table 3. Gibbs free energy (binding affinity) and its components for the training set of NQO1 inhibitors COU1-22 [4].

Training Set								
New id.	Old id. [4]	IC ₅₀ ^{exp} (nM)	M _w [g.mol ⁻¹]	$\Delta\Delta H_{MM}$ [kca.mol ⁻¹]	$\Delta\Delta G_{sol}$ [kca.mol ⁻¹]	$\Delta\Delta TS_{vib}$ [kca.mol ⁻¹]	$\Delta\Delta G_{com}$ [kca.mol ⁻¹]	pIC ₅₀ ^{exp}
COU1	12	0.18	436	0.00	0.00	0.00	0.00	9.74
COU2	1	2.60	336	3.32	1.29	0.40	4.21	8.59
COU3	2	14	364	8.76	-2.21	1.34	5.22	7.85
COU4	3	2.80	396	2.28	1.10	1.11	2.27	8.55
COU5	4	11	396	4.41	0.74	0.68	4.47	7.96
COU6	6	4.50	372	2.48	-0.59	-2.15	4.03	8.35
COU7	7	3.80	372	6.35	-3.52	0.44	2.39	8.42
COU8	10	0.41	392	0.01	1.75	3.91	-2.14	9.39
COU9	13	0.42	392	0.15	-0.44	0.26	-0.55	9.38
COU10	14	1221	408	14.24	-4.37	-0.80	10.66	5.91
COU11	15	588	378	13.79	-1.46	2.54	9.78	6.23
COU12	16	7.70	318	9.48	-2.02	3.93	3.53	8.11
COU13	17	2.50	318	6.73	-1.35	3.55	1.83	8.60
COU14	18	39	280	10.58	-1.59	2.74	6.24	7.41
COU15	20	2.20	352	2.56	0.66	2.36	0.86	8.66

Training Set								
New id.	Old id. [4]	IC ₅₀ ^{exp} (nM)	M _w [g.mol ⁻¹]	ΔΔH _{MM} [kca.mol ⁻¹]	ΔΔG _{sol} [kca.mol ⁻¹]	ΔΔTS _{vib} [kca.mol ⁻¹]	ΔΔG _{com} [kca.mol ⁻¹]	pIC ₅₀ ^{exp}
COU16	21	35	302	11.6	-2.63	2.04	6.39	7.46
COU17	24	15	302	6.79	-1.86	1.66	3.27	7.82
COU18	25	24	302	6.47	0.91	3.08	4.30	7.62
COU19	26	14	302	7.89	-0.67	2.61	4.61	7.85
COU20	27	144	252	12.15	-1.84	2.12	8.19	6.84
COU21	28	31	280	9.92	-1.15	3.58	5.20	7.51
COU22	29	9.90	308	8.44	-1.02	4.51	2.91	8.00

To further elucidate COUs' binding affinity to NQO1, the gas-phase complexation enthalpy (ΔΔH_{MM}) was initially computed and analyzed by correlating it with pIC₅₀^{exp}. The statistical significance of this linear relationship (see Table 5, equation A) underscored the crucial role of inhibitor-enzyme interactions (ΔΔH_{MM}), particularly when solvent effects and entropy loss upon binding to NQO1 were neglected. This correlation explained approximately 85% of the variation in pIC₅₀^{exp} values, highlighting the enthalpic contribution to ligand binding affinity.

Additionally, a more comprehensive descriptor, the GFE of NQO1 complex formation, incorporating all key components ΔΔH_{MM}, ΔΔTS_{vib}, and ΔΔG_{sol} was evaluated (see Table 5,

equation B). The relatively high regression coefficient (R²) demonstrated that structural insights derived from 3D models of NQO1 complexes could reliably predict the NQO1 inhibitory potency of novel COU analogs (with a similar binding mode) using the QSAR B model, as shown in Table 3.

Binding mode of COUs: The inhibitors (COUs) investigated in this study belong to a newly synthesized series of Coumarin derivatives, as detailed in reference [4]. Coumarins are well known for their metal ion-chelating properties, with their acidic functional group playing a crucial role in NQO1 inhibition. The active site was analyzed using X-ray crystallographic data of NQO1 (PDB code: 3JSX) complexed with one of the most potent inhibitors identified in this study.

Table 4. Gibbs free energy (binding affinity) and its components for the validation set inhibitors VCOU1-6 [4].

Validation Set										
New id.	Old id.[4]	IC ₅₀ ^{exp} (nM)	pIC ₅₀ ^{exp}	M _w [g.mol ⁻¹]	ΔΔH _{MM} [kca.mol ⁻¹]	ΔΔG _{sol} [kca.mol ⁻¹]	ΔΔTS _{vib} [kca.mol ⁻¹]	ΔΔG _{com} [kca.mol ⁻¹]	pIC ₅₀ ^{pre}	pIC ₅₀ ^{pre} / pIC ₅₀ ^{exp}
VCOU1	5	6.00	8.22	396	2.47	0.68	0.32	2.83	8.34	1.01
VCOU2	8	9.00	8.05	405	2.74	-0.41	-2.97	5.30	7.64	0.95
VCOU3	9	4.80	8.32	652	-2.34	-3.13	-8.78	3.30	8.21	0.99
VCOU4	19	6.30	8.20	352	6.86	-0.56	2.67	3.63	8.11	0.99
VCOU5	22	6.30	8.20	352	6.05	-0.91	2.04	3.11	8.26	1.01
VCOU6	23	6.00	8.22	352	7.65	-2.06	1.36	4.24	7.94	0.97

^a for the chemical structures of the training set of inhibitors see Table 1; ^b M_w is the molar mass of inhibitors; ^c ΔΔH_{MM} is the relative enthalpic contribution to the GFE change related to E-I complex formation derived by MM; ΔΔH_{MM} ≈ [E_{MM}{E-I_x} - E_{MM}{I_x}] - [E_{MM}{E-I_{ref}} - E_{MM}{I_{ref}}], I_{ref} is the reference inhibitor COU12; ^d ΔΔG_{sol} is the relative solvent effect contribution to the GFE change of E-I complex formation: ΔΔG_{sol} = [G_{sol}{E-I_x} - G_{sol}{I_x}] - [G_{sol}{E-I_{ref}} - G_{sol}{I_{ref}}]; ^e -ΔΔTS_{vib} is the relative entropic contribution of inhibitor to the GFE of E-I_x complex formation: ΔΔTS_{vib} = [TS_{vib}{I_x}E - TS_{vib}{I_x}] - [TS_{vib}{I_{ref}}E - TS_{vib}{I_{ref}}]; ^f ΔΔG_{com} is the overall relative GFE change of E-I_x complex formation: ΔΔG_{com} ≈ ΔΔH_{MM} + ΔΔG_{sol} - ΔΔTS_{vib}; ^g K_i^{exp} is the experimental inhibitory concentration of NQO1 obtained from ref. [4]; ^h ratio of predicted and experimental half-maximal inhibition concentrations pIC₅₀^{pre} / pIC₅₀^{exp} (pIC₅₀^{pre} = -log₁₀IC₅₀^{pre}) was predicted from computed ΔΔG_{com} using the regression equation for NQO1 shown in Table 3 B.

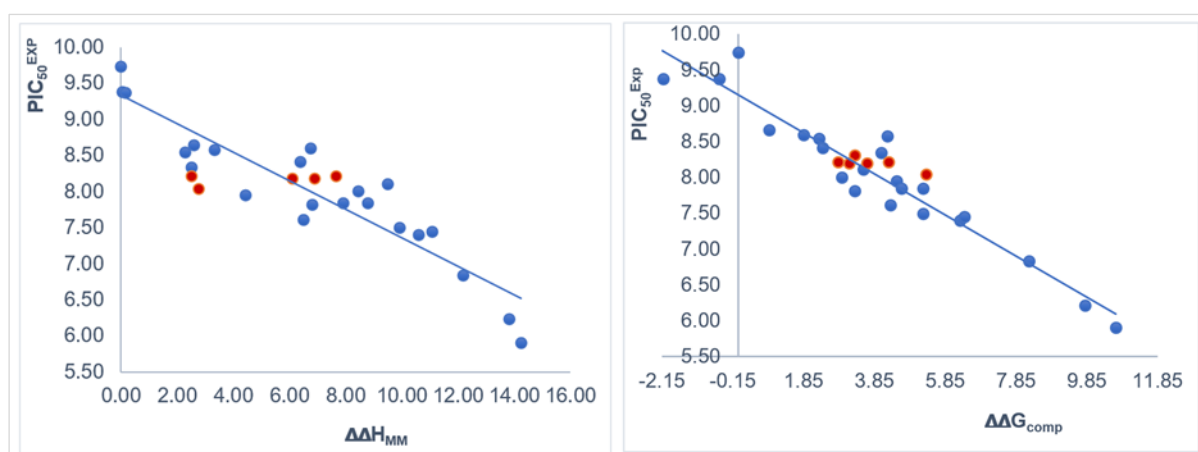
Table 5. Analysis of computed binding affinities ($\Delta\Delta G_{\text{com}}$), its enthalpic component $\Delta\Delta H_{\text{MM}}$, and experimental inhibitory concentrations $pIC_{50}^{\text{exp}} = -\log_{10} IC_{50}^{\text{exp}}$ of COUs towards NQO1 [4].

Statistical Data of Linear Regression	A	B
$pIC_{50}^{\text{exp}} = -0,1981 \times \Delta\Delta H_{\text{MM}} + 9,3435$	A	
$pIC_{50}^{\text{exp}} = -0,2867 \times \Delta\Delta G_{\text{com}} + 9,1546$		B
Number of compound n	22	22
Squared correlation coefficient of	0.85	0.91
LOO cross-validated Squared	0.86	0.94
Standard error of regression σ	0,23	0.14
Statistical significance of regression.	224.1	584.3
Level of statistical significance α	> 95	> 95
Range of activities IC_{50}^{exp} [nM]	0.18 –1221	

Interaction Energy: Key structural insights were obtained from the interaction energy (IE, ΔE_{int}) diagram generated for each inhibitor in the training set. The decomposition of IE into contributions from specific NQO1 active site residues provided valuable guidance for selecting relevant R groups at sites to enhance the binding affinity of COU analogs, thereby improving ligand potency. A comparative analysis of the IE values calculated for COUs, classified into three levels of activity (highest, moderate and lowest), identified residues for which it would be possible to increase the contributions to binding affinity. However, the results indicated that the IE contributions from active site residues remained consistent across all three classes of inhibitors, and no viable substitu-

tions were identified to enhance binding affinity, unlike previous findings for thymine-like inhibitors of NQO1.

The statistical data depicted in Table 5 confirmed the validity of the correlation Equations (A) and (B). Likewise, the ratio $pIC_{50}^{\text{pre}}/IC_{50}^{\text{exp}}$ close to one (1) for the validation set, where the pIC_{50}^{pre} values were estimated with correlation Equation (B), exhibits the substantial predictive power of the complexation QSAR model as showed in Table 3. Thus, the regression Equation (B) and the computed $\Delta\Delta G_{\text{com}}$ GFEs can be used for the prediction of the inhibitory potencies pIC_{50}^{pre} against NQO1 for novel COU analogs, provided they share the same binding mode as the training set COU1-22.

**Figure 1.** (Left) plot of correlation equation between pIC_{50}^{exp} and relative enthalpic contribution to the GFE ($\Delta\Delta H_{\text{MM}}$ [kcal.mol⁻¹]). (Right) similar plot for relative complexation Gibbs free energies of the NQO1-COU complex formation $\Delta\Delta G_{\text{com}}$ [kcal.mol⁻¹] of the training set [9]. The validation set data points are shown in red color.

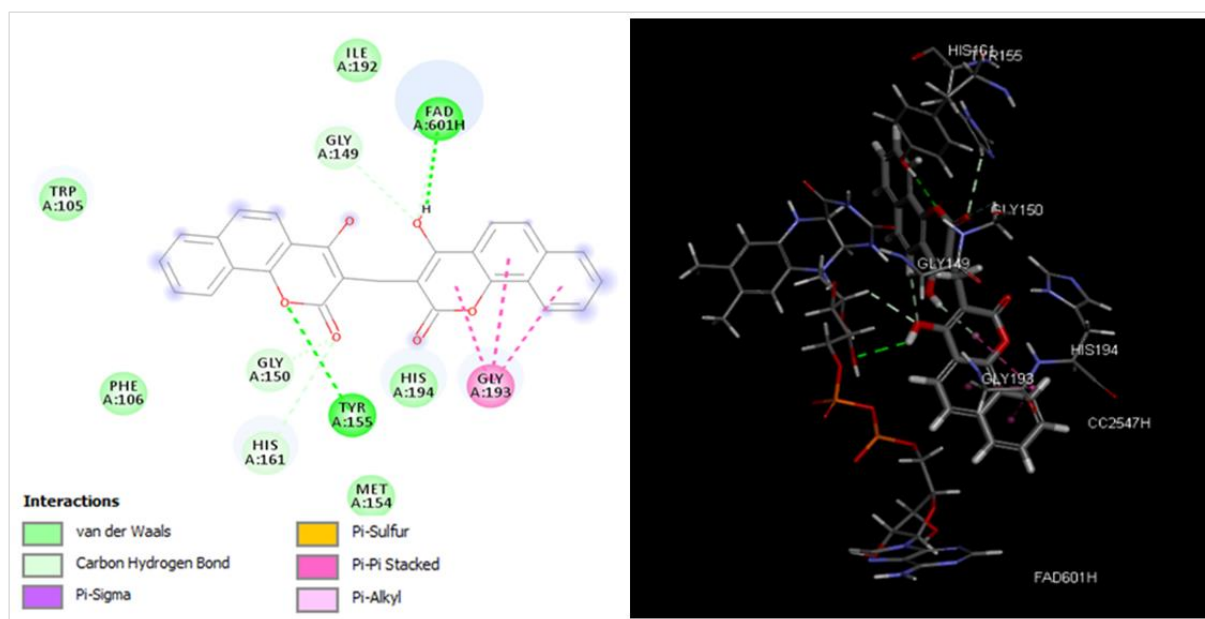


Figure 2. (Left) 2D schematic interaction diagram of the most potent inhibitor COU1 at the active site of NQO1 and (Right) 3D schematic interaction of COU1 at the enzyme active site.

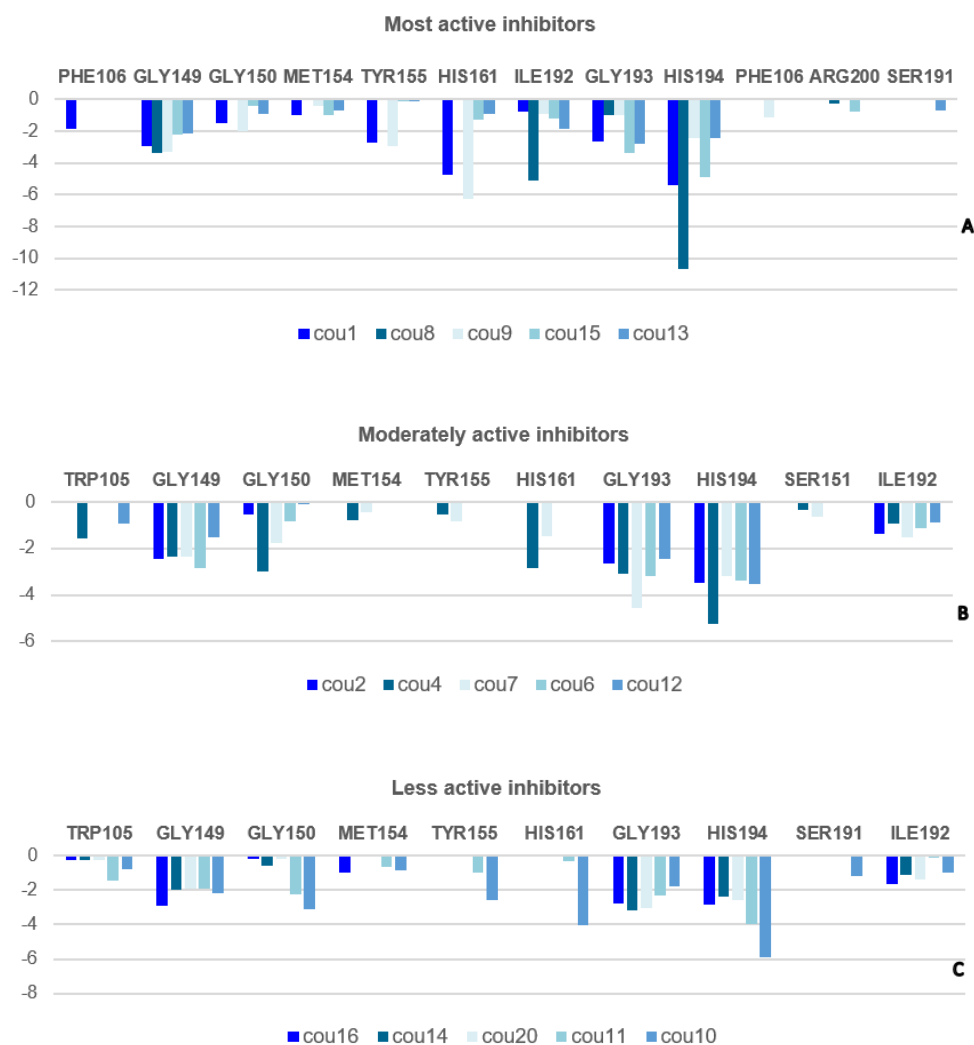


Figure 3. Molecular Mechanics intermolecular interaction energy E_{int} breakdown to residue contributions in [kcal.mol⁻¹]: (A: Top) the most active inhibitors, (B: Middle) moderately active inhibitors, (C: Bottom) less active inhibitors, Table 2 [4].

3.2. 3D-QSAR Pharmacophore Model

Generation and Validation of 3D-QSAR Pharmacophore: A 3D-QSAR pharmacophore model for NQO1 inhibition was developed using the active conformations of the 22 inhibitors originating from the trainings set and validated with the 6 inhibitors of the validation set. These inhibitors spanned a broad range of experimental activities (0,18–1221 nM), covering nearly three orders of magnitude. The pharmacophore generation process followed three key steps: (i) the constructive step, (ii) the subtractive step, and (iii) the optimization step [16], as previously described [19].

Hypotheses were evaluated based on errors in activity estimates from regression and complexity using a simulated annealing approach. The top 10 unique pharmacophore hypotheses were retained, all exhibiting five-point features, with the corresponding data listed in Table 6. Their selection was

based on significant statistical parameters, including a high correlation coefficient, low total cost, and low RMSD. The cost difference ($\Delta = 986.56$) was calculated as follows: null cost (1038.78) – fixed cost (52.14), indicating a high probability (>90%) that the model represents a true correlation [24].

The evaluation of hypothesis 1 (Hypo1) involves mapping the most active compound from the training set, COU1 (Figure 4D), illustrating the geometry of the Hypo1 pharmacophore for NQO1 inhibition. The following regression equation correlating pIC_{50}^{exp} as a function of pIC_{50}^{pre} for Hypo1 is $pIC_{50}^{exp} = 0.9092 \times pIC_{50}^{pre} + 0.7386$. This relationship is plotted in Figure 4E ($n = 22$, $R^2 = 0.91$, $R^2_{cv} = 0.90$, F-test = 191, $\sigma = 0.30$, $\alpha > 95\%$). These results indicate that the PH4 model is highly reliable and could be efficiently used to identify new COU analogs.

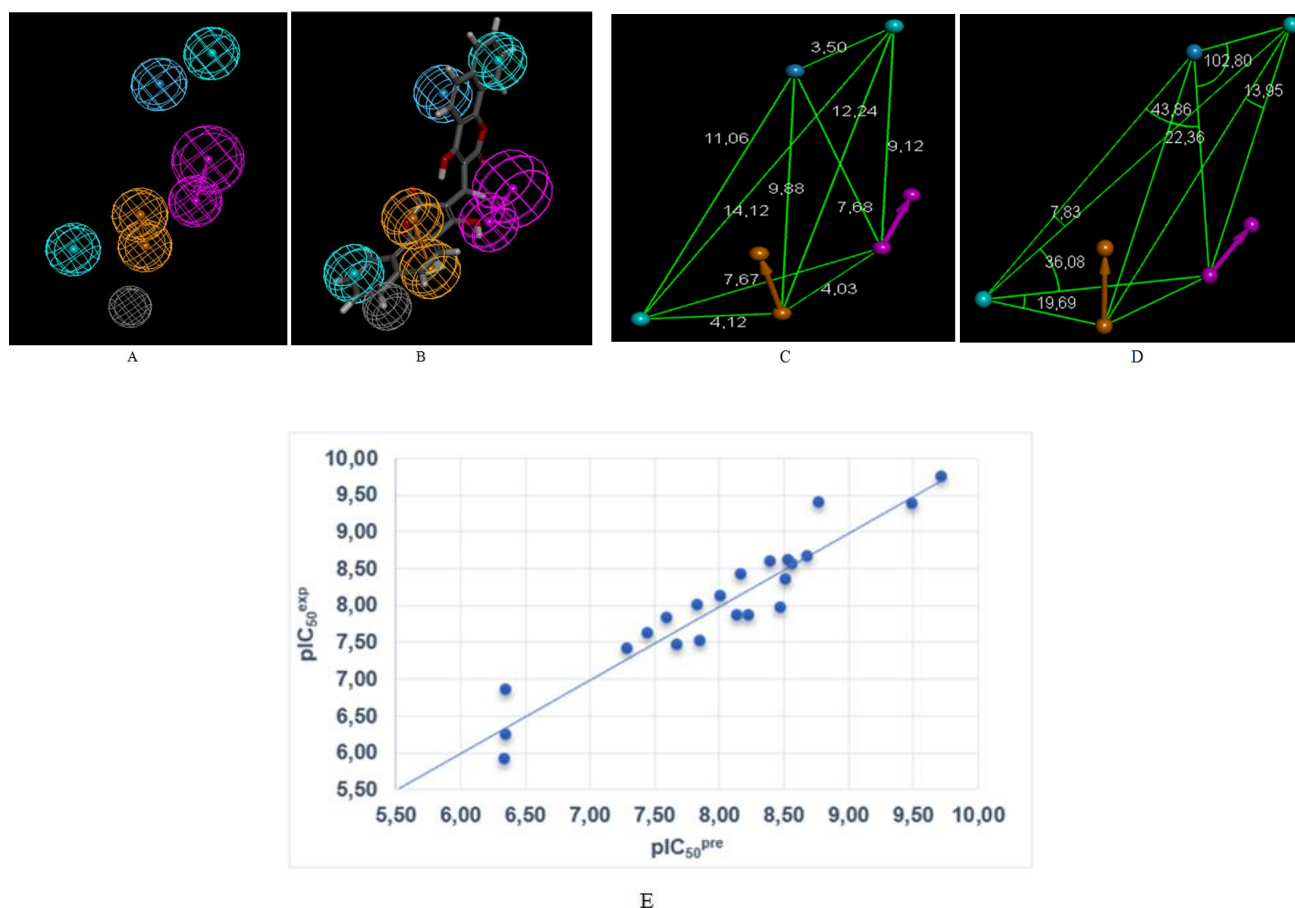


Figure 4. (A) Features coordinates of centers, (B) mapping of pharmacophore of NQO1 inhibitor with the most potent molecule COU1, (C) Distances between centers, (D) angles between centers of pharmacophoric features. Feature legend: HYD = Hydrophobic (cyan), HBA = Hydrogen bond Acceptor (green), HBD = Hydrogen bond Donor (pink), Excluded volume. (E) Correlation plot of experimental vs. predicted inhibitory activity.

Table 6. Parameters of 10 generated PH4 pharmacophoric hypotheses for NQO1 inhibitor after Cat-Scramble validation procedure (49 scrambled runs for each hypothesis at the selected level of confidence of 98%).

Hypothesis	RSMD ^a	R ² ^b	Total Cost ^c	Costs Difference ^d	Closest Random ^e	Features ^f
Hypo 1	2.896	0.95	145.5	893.2	192.1	HBD, HYD-Ar, HYD, HYD, R-Ar
Hypo 2	3.314	0.94	173.0	865.7	314.6	HBD, HYD-Ar, HYD, HYD, R-Ar
Hypo 3	3.416	0.93	180.5	858.2	351.2	HBD, HYD-Ar, HYD, HYD, R-Ar
Hypo 4	3.618	0.93	196.2	842.5	388.4	HBD, HYD-Ar, HYD-Ar, HYD, R-Ar
Hypo 5	3.634	0.92	197.4	841.3	425.7	HBD, HYD, HYD, HYD, R-Ar
Hypo 6	3.937	0.91	222.7	816.0	426.5	HBD, HYD, HYD, HYD, R-Ar
Hypo 7	4.004	0.90	228.5	810.2	439.1	HBD, HYD-Ar, HYD, HYD, R-Ar
Hypo 8	4.069	0.91	234.2	804.5	459.5	HBD, HYD-Ar, HYD, HYD, R-Ar
Hypo 9	4.569	0.88	282.2	756.5	479.4	HBD, HYD-Ar, HYD-Ar, HYD, HYD
Hypo 10	4.659	0.87	291.7	747.0	520.3	HBD, HYD-Ar, HYD-Ar, HYD
Fixed Cost	0	0	52.14			
Null Cost	9.53	0	1038.78			

Configuration cost = 12.09.

^a root mean squared deviation; ^b squared correlation coefficient; ^c overall cost parameter of PH4 pharmacophore; ^d cost difference between Null cost and hypothesis total cost; ^e lowest cost from 49 scrambled runs at a selected level of confidence of 98%. ^f HBA (hydrogen-bond acceptor); HYD (hydrophobic); HYD-Ar (hydrophobic aromatic)

3.3. Virtual Screening

Virtual screening of combinatorial libraries has emerged as a powerful approach for identifying potential hit compounds. Our previous studies on inhibitor design have demonstrated its effectiveness in selecting promising candidates by exploring vast chemical spaces and predicting key molecular interactions [25-30].

Virtual Library

A preliminary virtual library (VL) was generated by modifying the COU scaffold at positions R₁, R₂, R₃, and R₄, as outlined in Table 7. During the enumeration process, the R-groups listed in Table 7 were systematically attached to in positions R₁ to R₄ of the COU scaffold to form a combinatorial library of the size: $R_1 \times R_2 \times R_3 \times R_4 = 16 \times 16 \times 16 \times 16 = 65,536$ analogs. All generated analogs maintained the substitution pattern of the most potent inhibitor, COU1.

To construct this library, fragments were sourced from chemical databases of available compounds [31]. To enhance

drug-like properties and reduce the library size, we applied a series of selection filters. These included the Lipinski rule-of-five, which, for example, excluded compounds with molecular weights above 500 g/mol [32]. This refinement resulted in a more focused library, better suited for subsequent in silico screening.

3.4. In Silico Screening of Library of COUs

The focused library of 65,536 analogs was further screened for molecular structures that align with the 3D-PH4 pharmacophore model Hypo1 for NQO1 inhibition. A total of 63 COU analogs matched at least four features of the pharmacophore. These top-ranking analogs (PH4 hits) were then subjected to complexation QSAR model screening. The computed Gibbs free energy (GFE) of NQO1-COU complex formation, along with its components and the predicted half-maximal inhibitory concentrations (IC_{50}^{pre}), derived from correlation Equation B (Table 3), are summarized in Table 8.

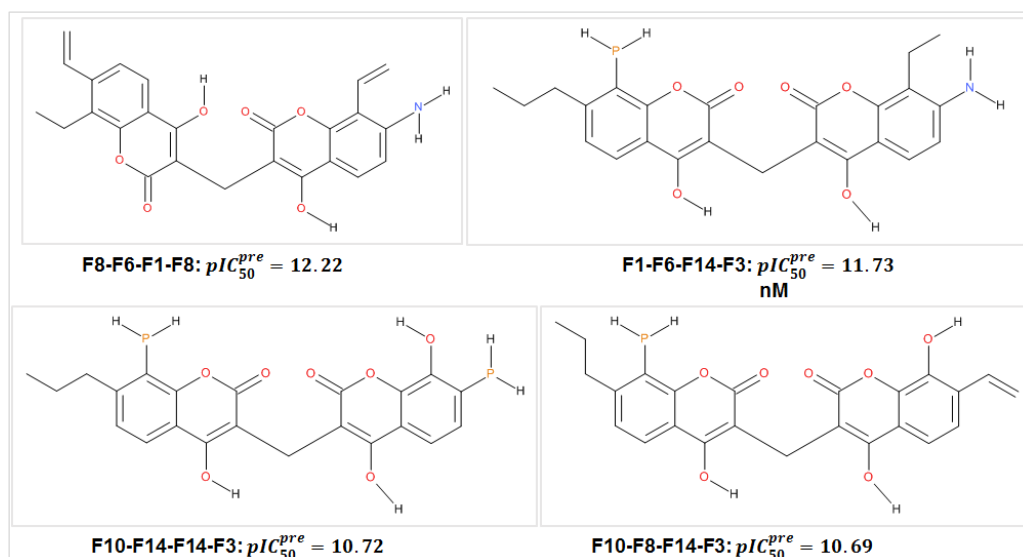


Figure 5. Best COU Analogs with scaffold of NQO1, the name is concatenation.

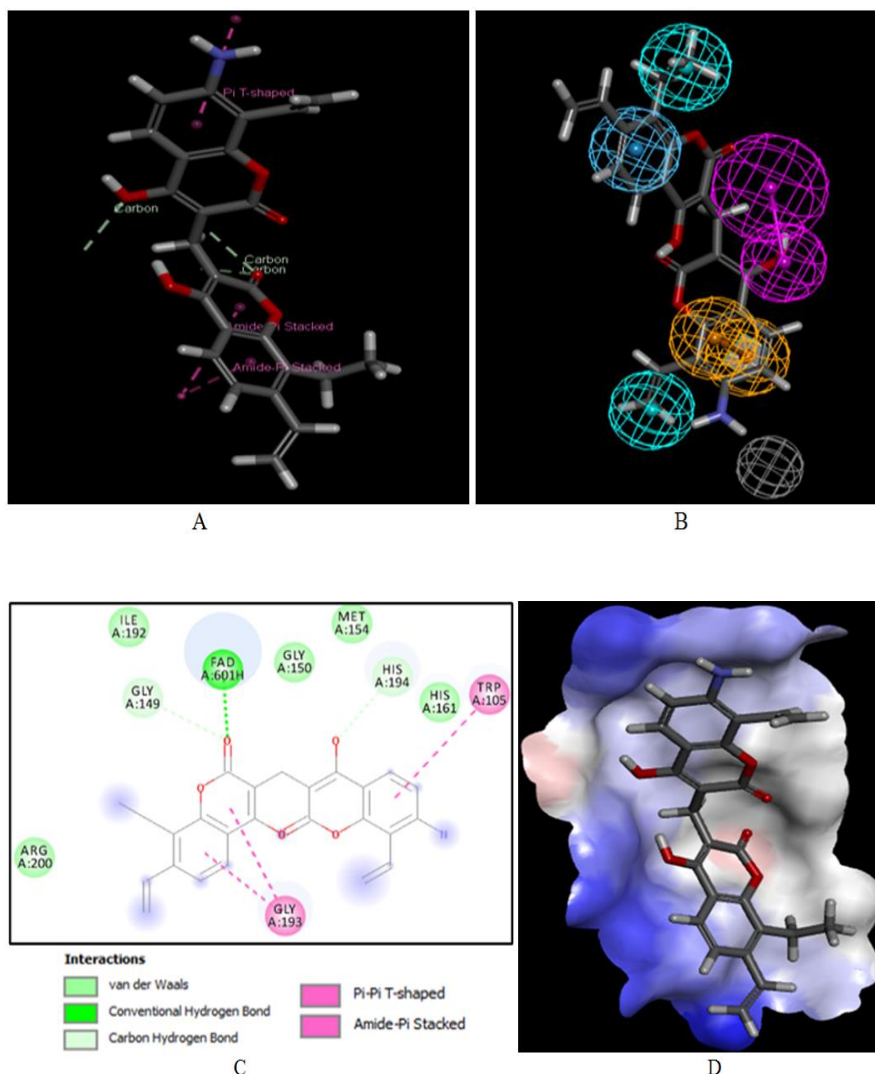
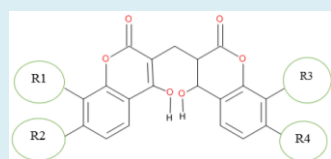
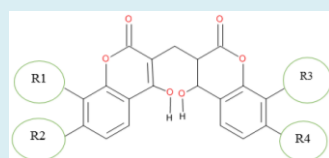


Figure 6. (A) Close up of virtual hit F8-F6-F1-F8, the most active designed COU analog ($pIC_{50}^{pre} = 12.22$) at the active site of NQO1. (B) Mapping of the COU F8-F6-F1-F8 to NQO1 inhibition pharmacophore. (C) 2D schematic interaction diagram of the best active designed COU analog F8-F6-F1-F8 at the active site of NQO1. (D) Surface of the active site of NQO1 with bound best active designed COU analog. The binding site surface is colored according to residue hydrophobicity: red = hydrophobic, blue = hydrophilic, and white = intermediate.

Table 7. R1, R2, R3 and R4-groups (fragments, building blocks, substituents) used in the design of the initial diversity virtual combinatorial library.**New analogues**

C 1 F	3-[(7-amino-4-hydroxy-2-oxo-8-vinyl-chromen-3-yl)methyl]-8-ethyl-4-hydroxy-7-vinyl-chromen-2-one	C 2 F	3-[(8-ethyl-4,7-dihydroxy-2-oxo-chromen-3-yl)methyl]-4-hydroxy-7-methyl-8-vinyl-chromen-2-one	C 3 F	3-[(8-ethyl-4-hydroxy-7-nitroso-2-oxo-chromen-3-yl)methyl]-4-methoxy-7-methyl-8-vinyl-chromen-2-one
C 4 F	3-[(7-acetyl-4-hydroxy-8-methyl-2-oxo-chromen-3-yl)methyl]-7-ethyl-4-hydroxy-8-methyl-chromen-2-one	C 5 F	3-[(8-ethyl-4-hydroxy-2-oxo-7-vinyl-chromen-3-yl)methyl]-4-methoxy-8-methyl-7-vinyl-chromen-2-one	C 7 F	8-ethyl-4-hydroxy-3-[[4-methoxy-8-(methylamino)-2-oxo-7-vinyl-chromen-3-yl)methyl]-7-vinyl-chromen-2-one
C 9 F	3-[(8-ethyl-4-hydroxy-7-nitroso-2-oxo-chromen-3-yl)methyl]-4-methoxy-7-methyl-8-vinyl-chromen-2-one	C 10 F	3-[(7-amino-8-ethyl-4-hydroxy-2-oxo-chromen-3-yl)methyl]-4-hydroxy-8-phosphanyl-7-propyl-chromen-2-one	C 13 F	3-[(8-acetyl-4-hydroxy-2-oxo-7-vinyl-chromen-3-yl)methyl]-4-hydroxy-8-methyl-7-propyl-chromen-2-one
C 14 F	4-hydroxy-3-[(7-hydroxy-4-methoxy-2-oxo-8-vinyl-chromen-3-yl)methyl]-2-oxo-8-propyl-chromene-7-carboxylic acid	C 15 F	3-[(4,7-dihydroxy-8-methyl-2-oxo-chromen-3-yl)methyl]-4-hydroxy-2-oxo-7-vinyl-chromene-8-carboxylic acid; ethane	C 17 F	3-[(8-ethyl-4-hydroxy-2-oxo-7-vinyl-chromen-3-yl)methyl]-4-methoxy-8-methyl-2-oxo-chromene-7-carbaldehyde
C 18 F	4-hydroxy-3-[(4-hydroxy-7-methyl-2-oxo-8-propyl-chromen-3-yl)methyl]-2-oxo-8-vinyl-chromene-7-carbaldehyde	C 19 F	4-hydroxy-3-[(4-hydroxy-8-methyl-2-oxo-7-phosphanyl-chromen-3-yl)methyl]-2-oxo-7-propyl-chromene-8-carboxamide	C 20 F	3-[(8-acetyl-4-hydroxy-2-oxo-7-vinyl-chromen-3-yl)methyl]-4-methoxy-8-methyl-7-vinyl-chromen-2-one
C 22 F	7-acetyl-4-hydroxy-3-[(7-hydroxy-4-methyl-2-oxo-8-phosphanyl-chromen-3-yl)methyl]-8-propyl-chromen-2-one	C 25 F	4-hydroxy-3-[(4-hydroxy-8-methyl-7-nitro-2-oxo-chromen-3-yl)methyl]-7-methyl-8-sulfanyl-chromen-2-one	C 26 F	8-ethyl-4-hydroxy-3-[(4-methoxy-7,8-dimethyl-2-oxo-chromen-3-yl)methyl]-2-oxo-chromene-7-carbaldehyde
C 27 F	4-hydroxy-3-[(4-hydroxy-2-oxo-8-phosphanyl-7-propyl-chromen-3-yl)methyl]-7-methyl-8-vinyl-chromen-2-one	C 28 F	7-ethyl-4-hydroxy-3-[(4-hydroxy-2-oxo-8-phosphanyl-7-propyl-chromen-3-yl)methyl]-2-oxo-chromene-8-carboxamide	C 29 F	8-(1-aminovinyl)-4-hydroxy-3-[(4-hydroxy-2-oxo-7,8-divinyl-chromen-3-yl)methyl]-7-methyl-chromen-2-one; hydrate
C 30 F	3-[(7-ethyl-4-hydroxy-2-oxo-8-vinyl-chromen-3-yl)methyl]-4-hydroxy-2-oxo-7-phosphanyl-chromene-8-carboxylic acid	C 31 F	8-ethyl-3-[(7-hydrosulfinyl-4-hydroxy-8-isopropenyl-2-oxo-chromen-3-yl)methyl]-4-hydroxy-7-sulfanyl-chromen-2-one	C 33 F	4,7-dihydroxy-3-[(4-hydroxy-2-oxo-7-phosphanyl-8-propyl-chromen-3-yl)methyl]-8-vinyl-chromen-2-one
C 34 F	3-[(7-amino-8-hydrosulfinyl-4-hydroxy-2-oxo-chromen-3-yl)methyl]-4-hydroxy-7-methyl-8-vinyl-chromen-2-one	C 35 F	4,7-dihydroxy-3-[(4-hydroxy-2-oxo-8-propyl-7-sulfanyl-chromen-3-yl)methyl]-8-phosphanyl-chromen-2-one	C 36 F	8-acetyl-3-[(8-amino-4-hydroxy-2-oxo-7-vinyl-chromen-3-yl)methyl]-7-ethyl-4-hydroxy-chromen-2-one
C 37 F	7-ethyl-3-[(7-formyl-4-hydroxy-2-oxo-8-vinyl-chromen-3-yl)methyl]-4-hydroxy-2-oxo-chromene-8-carboxylic acid	C 38 F	4-hydroxy-3-[(4-hydroxy-2-oxo-8-phosphanyl-7-propyl-chromen-3-yl)methyl]-2-oxo-7-sulfanyl-chromene-8-carboxylic acid	C 39 F	ammonia; 3-[(8-ethyl-4-hydroxy-2-oxo-7-vinyl-chromen-3-yl)methyl]-4-methoxy-7-methyl-8-vinyl-chromen-2-one
C 40 F	4,7-dihydroxy-3-[(4-hydroxy-8-nitroso-2-oxo-7-sulfanyl-chromen-3-yl)methyl]-8-vinyl-chromen-2-one	C 41 F	8-ethyl-4-hydroxy-3-[(7-hydroxy-4-methyl-2-oxo-8-vinyl-chromen-3-yl)methyl]-7-nitroso-chromen-2-one	C 42 F	3-[(7-amino-4-hydroxy-2-oxo-8-phosphanyl-7-propyl-chromen-3-yl)methyl]-4-hydroxy-2-oxo-7-vinyl-chromene-8-carbaldehyde



New analogues

C 4 3 F	3-[(7-acetyl-4-hydroxy-2-oxo-8-vinyl-chromen-3-yl)methyl]-4-hydroxy-8-nitroso-7-propyl-chromen-2-one	C 4 5 F	8-formyl-3-[(4-hydroxy-8-methyl-2-oxo-7-vinyl-chromen-3-yl)methyl]-4-methoxy-2-oxo-chromene-7-carboxamide	C 4 6 F	3-[(4,7-dihydroxy-2-oxo-8-vinyl-chromen-3-yl)methyl]-8-hydrosulfinyl-4-hydroxy-7-propyl-chromen-2-one
C 5 1 F	3-[(8-ethyl-4-hydroxy-7-nitroso-2-oxo-chromen-3-yl)methyl]-4-methoxy-7-methyl-8-vinyl-chromen-2-one	C 5 2 F	4-hydroxy-3-[(4-hydroxy-2-oxo-8-phosphanyl-7-sulfonyl-chromen-3-yl)methyl]-2-oxo-8-vinyl-chromene-7-carbaldehyde	C 5 3 F	3-[(7-ethyl-4-hydroxy-2-oxo-8-sulfonyl-chromen-3-yl)methyl]-4-hydroxy-2-oxo-8-vinyl-chromene-7-carboxylic
C 5 4 F	7-ethyl-3-[(7-formyl-4-hydroxy-2-oxo-8-vinyl-chromen-3-yl)methyl]-4-hydroxy-2-oxo-chromene-8-carboxylic	C 5 5 F	3-[(7-amino-4-hydroxy-2-oxo-8-vinyl-chromen-3-yl)methyl]-4-hydroxy-7-methyl-8-vinyl-chromen-2-one	C 5 6 F	4-hydroxy-3-[(4-hydroxy-2-oxo-8-sulfonyl-7-vinyl-chromen-3-yl)methyl]-2-oxo-chromene-7,8-dicarbaldehyde
C 5 7 F	3-[(4,7-dihydroxy-8-methyl-2-oxo-chromen-3-yl)methyl]-4,7-dihydroxy-2-oxo-chromene-8-carbaldehyde	C 5 8 7	3-[(4,8-dihydroxy-2-oxo-7-vinyl-chromen-3-yl)methyl]-4-hydroxy-8-phosphanyl-7-propyl-chromen-2-one	C 6 0 F	3-[(4,8-dihydroxy-2-oxo-7-phosphanyl-chromen-3-yl)methyl]-4-hydroxy-8-phosphanyl-7-propyl-chromen-2-one
C 6 1 F	3-[(8-formyl-4-hydroxy-2-oxo-7-phosphanyl-chromen-3-yl)methyl]-4-hydroxy-2-oxo-8-phosphanyl-chromene-7-carbaldehyde	C 6 3 F	3-[(4,8-dihydroxy-7-methyl-2-oxo-chromen-3-yl)methyl]-4-hydroxy-7-methyl-8-vinyl-chromen-2-one	C 6 4 F	3-[(4,8-dihydroxy-2-oxo-7-phosphanyl-chromen-3-yl)methyl]-4-hydroxy-8-nitroso-7-phosphanyl-chromen-2-one
C 7 3 F	3-[(7-formyl-4-hydroxy-2-oxo-8-sulfonyl-chromen-3-yl)methyl]-4,8-dihydroxy-2-oxo-chromene-7-carbaldehyde	C 7 6 F	4-hydroxy-3-[(4-hydroxy-2-oxo-8-phosphanyl-7-propyl-chromen-3-yl)methyl]-7-methyl-8-vinyl-chromen-2-one	C 7 8 F	7-ethyl-4-hydroxy-3-[(4-hydroxy-2-oxo-8-phosphanyl-7-propyl-chromen-3-yl)methyl]-2-oxo-chromene-8-carboxamide
C 8 1 F	3-[(8-ethyl-4,7-dihydroxy-2-oxo-chromen-3-yl)methyl]-4-hydroxy-8-nitroso-7-vinyl-chromen-2-one	C 8 2 F	7-ethyl-4-hydroxy-3-[(4-hydroxy-8-nitroso-2-oxo-7-phosphanyl-chromen-3-yl)methyl]-8-vinyl-chromen-2-one	C 8 3 F	4-hydroxy-3-[(4-hydroxy-8-methyl-2-oxo-7-propyl-chromen-3-yl)methyl]-2-oxo-8-phosphanyl-chromene-7-carboxylic
C 8 5 F	3-[(4,7-dihydroxy-2-oxo-8-vinyl-chromen-3-yl)methyl]-7-ethyl-4-hydroxy-8-nitroso-chromen-2-one	C 8 8 9 F	3-[(4,7-dihydroxy-8-nitroso-2-oxo-chromen-3-yl)methyl]-4-hydroxy-8-phosphanyl-7-propyl-chromen-2-one	C 9 1 F	4-hydroxy-3-[(4-hydroxy-2-oxo-7-propyl-8-sulfonyl-chromen-3-yl)methyl]-2-oxo-8-phosphanyl-chromene-7-carboxylic
C 9 2 F	3-[(8-acetyl-4-hydroxy-7-nitroso-2-oxo-chromen-3-yl)methyl]-4-methoxy-7-methyl-8-phosphanyl-chromen-2-one	C 9 3 F	4-hydroxy-3-[(4-hydroxy-2-oxo-8-phosphanyl-7-propyl-chromen-3-yl)methyl]-2-oxo-7-sulfonyl-chromene-8-carboxylic	C 9 6 F	3-[(4,7-dihydroxy-2-oxo-8-vinyl-chromen-3-yl)methyl]-4-hydroxy-7-propyl-8-sulfonyl-chromen-2-one
C 9 8 F	7-ethyl-4-hydroxy-3-[(4-hydroxy-2-oxo-7-phosphanyl-8-sulfonyl-chromen-3-yl)methyl]-8-vinyl-chromen-2-one	C 1 0 0	8-acetyl-4-hydroxy-3-[(4-hydroxy-2-oxo-7-propyl-8-sulfonyl-chromen-3-yl)methyl]-7-methyl-chromen-2-one	C 1 0 8 F	3-[(7-amino-8-hydrosulfinyl-4-hydroxy-2-oxo-chromen-3-yl)methyl]-4-hydroxy-7-propyl-8-sulfonyl-chromen-2-one

Table 8. GFE and their components for the top scoring 63 virtual COU analogs. The analog numbering concatenates the index of each substituent R_1 , R_2 , R_3 , and R_4 with the substituent numbers taken from Table 7.

N	Analog	M_w [g.mol ⁻¹]	$\Delta\Delta H_{MM}$ [kca.mol ⁻¹]	$\Delta\Delta G_{sol}$ [kca.mol ⁻¹]	$\Delta\Delta TS_{vib}$ [kca.mol ⁻¹]	$\Delta\Delta G_{com}$ [kca.mol ⁻¹]	pIC_{50}^{pre}
C1	F1-F8-F8-F6	431	3.55	-0.75	5.14	-2.35	9.83
C2	F1- F10- F8- F2	420	-0.08	1.97	2.41	-0.53	9.31
C3	F1-F13-F8-F10	435	4.79	-0.43	-0.37	4.73	7.80
C4	F2-F1-F2-F4	434	-1.36	2.51	5.85	-4.70	10.50
C5	F1-F8-F2-F9	432	4.18	-0.75	0.43	2.99	8.30
C7	F1-F8-F6-F8	458	3.23	-0.55	4.54	-1.85	9.69
C9	F1-F13-F8-F6	434	2.69	-0.47	2.12	0.11	9.12
C10	F1-F6-F14-F3	453	-3.41	3.17	8.74	-8.99	11.73
C13	F2-F3-F4-F8	460	-2.64	3.53	3.59	-2.70	9.93
C14	F3-F7-F8-F10	464	3.06	1.62	1.31	3.37	8.19
C15	F2-F10-F7-F8	436	-0.27	2.13	-1.29	3.16	8.25
C17	F2-F9-F1-F8	432	4.17	-0.74	0.40	3.04	8.28
C18	F3-F2-F8-F9	446	3.51	2.19	3.46	2.24	8.51
C19	F2-F14-F5-F3	467	-2.68	3.38	0.09	0.60	8.98
C20	F2-F8-F4-F8	444	2.11	-0.68	2.82	-1.39	9.55
C23	F3-F4-F14-F10	468	3.10	0.49	5.27	-1.68	9.64
C25	F2-F13-F16-F2	425	0.66	2.25	-1.33	4.24	7.94
C26	F2-F6-F1-F9	421	2.71	-0.19	0.38	2.14	8.54
C27	F8-F2-F14-F3	450	-2.84	2.99	2.06	-1.91	9.70
C28	F5-F1-F14-F3	481	-1.90	1.93	3.91	-3.87	10.26
C29	F5-F2-F8-F8	445	-0.96	3.26	3.19	-0.89	9.41
C30	F8-F1-F7-F14	466	-2.33	2.71	-17.93	18.32	3.90
C31	F4-F15-F1-F16	486	0.81	2.52	-0.36	3.69	8.10
C33	F3-F14-F8-F10	452	4.48	2.52	4.58	2.41	8.46
C34	F8-F2-F15-F6	439	1.81	1.64	1.55	1.89	8.61
C35	F3-F16-F14-F10	458	4.02	2.77	2.55	4.24	7.94
C36	F4-F1-F6-F8	447	-3.22	2.72	2.92	-3.43	10.14
C37	F7-F1-F8-F9	462	-2.04	2.19	-0.61	0.76	8.94
C38	F7-F16-F14-F3	486	3.59	3.14	1.47	-1.92	9.70
C39	F8-F6-F1-F8	431	-5.25	-0.37	5.06	-10.68	12.22
C40	F8-F10-F13-F16	439	0.75	3.00	1.07	2.68	8.39
C41	F8-F10-F1-F13	435	4.79	-0.42	-0.35	4.72	7.80
C42	F9-F8-F14-F6	437	-0.53	2.29	-0.58	2.34	8.48
C43	F8-F4-F13-F3	475	-2.49	2.68	-0.16	0.34	9.06
C45	F9-F5-F2-F8	447	3.32	-0.70	-1.13	3.75	8.08
C46	F8-F10-F15-F3	468	0.55	3.69	1.95	2.28	8.50

N	Analogs	M _w [g.mol ⁻¹]	ΔΔH _{MM} [kca.mol ⁻¹]	ΔΔG _{sol} [kca.mol ⁻¹]	ΔΔTS _{vib} [kca.mol ⁻¹]	ΔΔG _{com} [kca.mol ⁻¹]	pIC ₅₀ ^{pre}
C51	F8-F6-F1-F13	434	2.69	1.45	2.12	2.03	8.57
C52	F8-F9-F14-F16	454	1.02	1.98	-1.12	4.12	7.97
C53	F8-F7-F16-F1	466	-1.55	2.67	0.26	0.86	8.91
C54	F8-F9-F7-F1	462	-2.02	2.21	-1.21	1.40	8.75
C55	F8-F6-F8-F2	417	-0.35	2.65	1.46	0.84	8.91
C56	F9-F9-F16-F8	450	-0.03	2.42	-0.76	3.16	8.25
C57	F9-F10-F2-F10	410	3.25	2.14	-1.35	6.74	7.22
C58	F10-F8-F14-F3	452	-3.50	2.99	4.86	-5.37	10.69
C60	F10-F14-F14-F3	458	-3.54	3.19	5.10	-5.45	10.72
C61	F9-F14-F14-F9	456	-1.07	2.20	-1.61	2.74	8.37
C63	F10-F2-F8-F2	406	0.25	2.52	2.30	0.47	9.02
C64	F10-F14-F13-F14	445	-1.86	3.03	-0.26	1.43	8.74
C73	F10-F9-F16-F9	440	-0.49	4.20	-1.74	5.46	7.59
C76	F14-F3-F8-F2	450	-3.41	2.86	2.41	-2.95	10.00
C78	F14-F3-F5-F1	481	-5.56	4.72	2.80	-3.64	10.20
C81	F13-F8-F1-F10	435	-0.14	2.13	1.83	0.16	9.11
C82	F13-F14-F8-F1	451	-2.46	3.27	2.00	-1.18	9.49
C83	F14-F7-F2-F3	468	-3.17	2.78	1.53	-1.91	9.70
C85	F13-F1-F8-F10	435	1.70	2.50	0.77	3.43	8.17
C89	F13-F10-F14-F3	455	-0.90	2.94	3.79	-1.74	9.65
C91	F14-F7-F16-F3	486	-2.64	2.74	0.06	0.03	9.15
C92	F14-F16-F4-F13	471	1.48	0.00	0.16	1.32	8.77
C93	F14-F3-F7-F16	486	-3.41	3.12	1.43	-1.71	9.65
C96	F16-F3-F8-F10	452	-1.15	2.77	1.99	-0.36	9.26
C98	F16-F14-F8-F1	454	-0.67	2.44	2.55	-0.78	9.38
C100	F16-F3-F4-F2	466	-1.67	3.14	3.77	-2.31	9.82
C108	F15-F6-F16-F3	473	-0.48	2.42	1.42	0.52	9.01
Ref	COU1	436	0	0	0	0	pIC ₅₀ ^{exp} = 9.74 ^g

^a M_w is molar mass of inhibitor; ^b ΔΔH_{MM} is the relative enthalpic contribution to the GFE change of the NQO1-COU complex formation ΔΔG_{com} (for details see footnote of Table 2); ^c ΔΔG_{sol} is the relative solvation GFE contribution to ΔΔG_{com}; ^d ΔΔTS_{vib} is the relative (vibrational) entropic contribution to ΔΔG_{com}; ^e ΔΔG_{com} is the relative Gibbs free energy change related to the enzyme-inhibitor NQO1-COU complex formation ΔΔG_{com} ≡ ΔΔH_{MM} + ΔΔG_{sol} - ΔΔTS_{vib}; ^f pIC₅₀^{pre} is the predicted inhibition potency towards NQO1 calculated from ΔΔG_{com} using correlation Equation B, Table 3; ^g pIC₅₀^{exp} [4] is given for the reference inhibitor COU1.

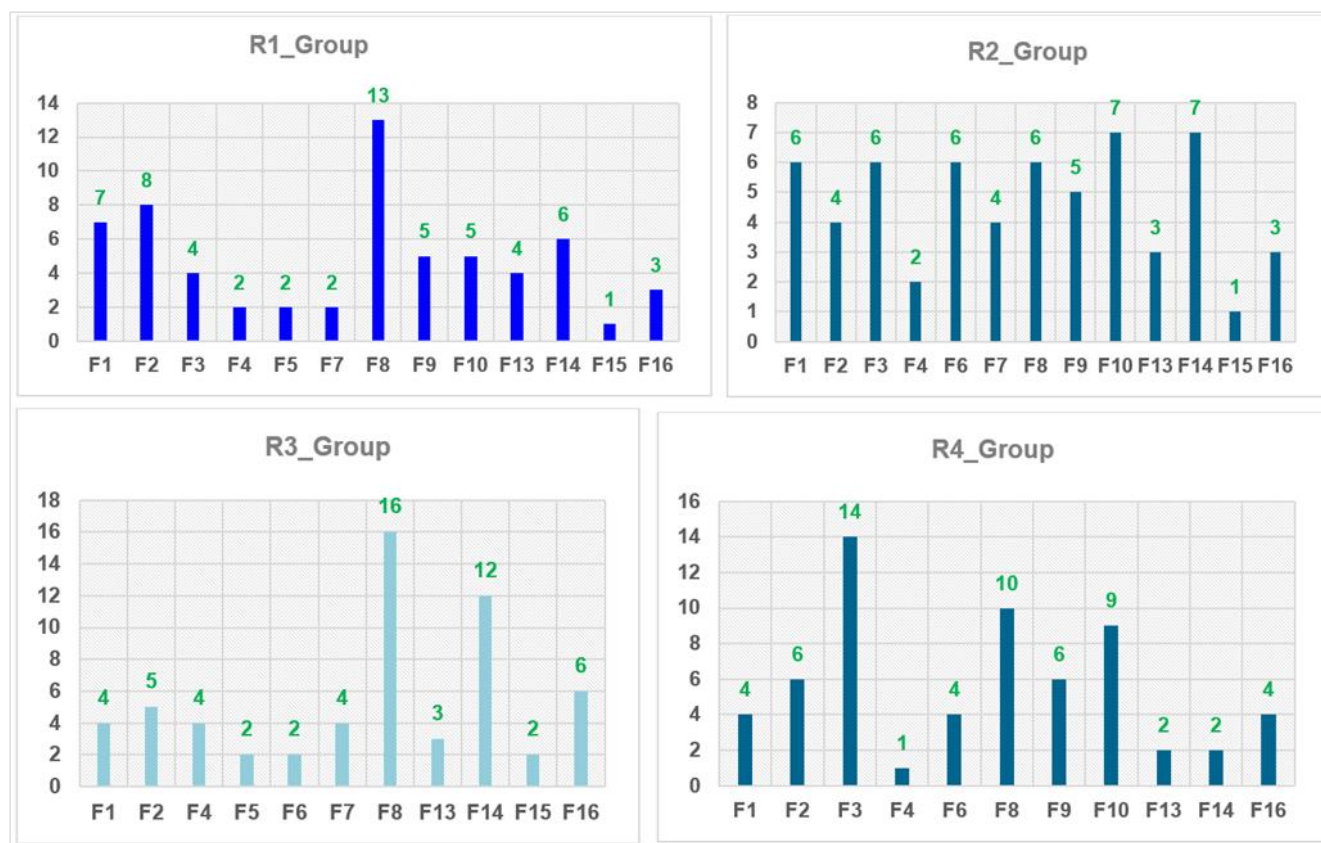


Figure 7. Histograms of frequency of occurrence of individual R-groups in the 63 best selected analogs mapping to four features of the PH4 pharmacophore hypothesis Hypo1.

Table 9. ADME-related properties of the best designed analogs and known anticancer agents either in clinical use or currently undergoing clinical testing computed by QikProp [22].

Analogues ^a	#stars ^b	M _w ^c	S _{mol} ^d	S _{molhfo} ^e	V _{mol} ^f	#rotB ^g	HB _{don} ^h
F1-F8-F8-F6	0	431	725.8	250.2	1299.1	8	3.5
F2-F1-F2-F4	0	434	726.5	358.7	1306.2	6	2
F1-F6-F14-F3	1	454	747.5	293.5	1345.1	9	5.5
F2-F3-F4-F8	0	460	768.5	367.3	1390.2	8	2
F5-F1-F14-F3	1	481	751.3	290.4	1378.3	9	6
F4-F1-F6-F8	0	447	702.3	273.6	1293.5	8	3.5
F8-F6-F1-F8	0	431	718.7	250.1	12978	8	3.5
F10-F8-F14-F3	1	452	730	241.1	1308	9	5
F10-F14-F14-F3	2	458	724	177	1289.2	9	7
F14-F3-F8-F2	1	450	714.4	301.6	1311.2	8	4
F14-F3-F5-F1	1	481	752.2	291.1	1379.3	9	6
F16-F3-F4-F2	0	466	734.5	322.6	1331.1	8	2.8
COU1	0	436	666.6	167.9	1201	7	4
Finast éide	0	372.550	605.996	431.904	1152.042	3	2.000
Alfuzosine	1	389.453	919.272	417.508	1140.426	3	3.000
T éraz (R)	0	387.438	621.508	422.153	1137.212	9	2.000

Analogues ^a	#stars ^b	M _w ^c	S _{mol} ^d	S _{molhfo} ^e	V _{mol} ^f	#rotB ^g	HB _{don} ^h
T éraz (S)	0	387.438	603.495	405.495	1131.925	5	2.000

Analogues ^a	HB _{acc} ⁱ	LogP _{o/w} ^j	LogS _{wat} ^k	LogK _{hsa} ^l	LogB/B ^m	BIP _{caco} ⁿ	#metab ^o	PIC ₅₀ ^{prep}	HOA ^q	%HOA ^r
F1-F8-F8-F6	7.5	2.845	-6.032	0.235	-2.063	119.2	5	9.83	3	80.8
F2-F1-F2-F4	8.5	2.651	-5.822	0.230	-1.870	135.2	6	10.50	3	80.6
F1-F6-F14-F3	7.5	2.878	-6.241	0.138	-1.993	117.2	7	11.73	2	80.8
F2-F3-F4-F8	8.5	3.290	-6.337	0.349	-1.928	173.6	5	9.93	3	86.3
F5-F1-F14-F3	9	2.330	-6.208	-0.007	-2.137	79.6	7	10.26	2	74.6
F4-F1-F6-F8	9.5	2.047	-5.621	-0.057	-1.936	119.9	6	10.14	3	76.1
F8-F6-F1-F8	7.5	2.841	-6.032	0.239	-2.018	120.8	5	12.22	3	80.8
F10-F8-F14-F3	7.3	3.015	-6.365	0.133	-1.812	158.5	6	10.69	3	84
F10-F14-F14-F3	7.3	2.639	-6.227	-0.106	-1.779	117	6	10.72	3	79.4
F14-F3-F8-F2	6.5	3.830	-6.706	0.396	-1.162	472.2	6	10.00	3	100
F14-F3-F5-F1	9	2.344	-6.208	-0.005	-2.129	81.2	7	10.20	2	74.9
F16-F3-F4-F2	9	2.827	-6.219	0.107	-1.687	190.1	6	9.82	3	84.3
COU1	9.3	1.641	-5.464	-0.364	-2.453	8.3	5		2	53.0
Finast éride	5.000	3.553	-4.91	0.549	-0.75	777.322	1		3	100
Alfuzosine	8.700	1.322	-3.639	-0.601	-1.076	347.334	5		3	80.158
T éraz (R)	9.200	1.273	-3.425	-0.452	-0.646	470.273	4		3	82.229
T éraz (S)	9.200	1.262	-3.425	-0.440	-0.596	467.119	4		3	82.113

^a designed COU analogs and known anticancer agents, Table 6; ^b drug likeness, number of property descriptors (24 out of the full list of 49 descriptors of QikProp, ver. 3.7, release 14) that fall outside of the range of values for 95% of known drugs; ^c molar mass in [g.mol⁻¹] (range for 95% of drugs: 300–500 g.mol⁻¹) [22]; ^d total solvent-accessible molecular surface, in [Å²] (probe radius 1.4 Å) (range for 95% of drugs: 300–1000 Å²); ^e hydrophobic portion of the solvent-accessible molecular surface, in [Å²] (probe radius 1.4 Å) (range for 95% of drugs: 0–750 Å²); ^f total volume of molecule enclosed by solvent-accessible molecular surface, in [Å³] (probe radius 1.4 Å) (range for 95% of drugs: 500–2000 Å³); ^g number of non-trivial (not CX3), non-hindered (not alkene, amide, small ring) rotatable bonds (range for 95% of drugs: 0–15); ^h estimated number of hydrogen bonds that would be donated by the solute to water molecules in an aqueous solution. Values are averages taken over a number of configurations, so they can assume non-integer values (range for 95% of drugs: 0.0–6.0); ⁱ estimated number of hydrogen bonds that would be accepted by the solute from water molecules in an aqueous solution. Values are averages taken over a number of configurations, so they can assume non-integer values (range for 95% of drugs: 2.0–20.0); ^j logarithm of partitioning coefficient between n-octanol and water phases (range for 95% of drugs: -2 to 6.5); ^k logarithm of predicted aqueous solubility, log S in [mol.dm⁻³] is the concentration of the solute in a saturated solution that is in equilibrium with the crystalline solid (range for 95% of drugs: -6.0 to 0.5); ^l logarithm of predicted binding constant to human serum albumin (range for 95% of drugs: -1.5 to 1.5); ^m logarithm of predicted brain/blood partition coefficient (range for 95% of drugs: -3.0 to 1.2); ⁿ predicted apparent Caco-2 cell membrane permeability in Boehringer-Ingelheim scale in [nm s⁻¹] (range for 95% of drugs: < 25 poor, > 500 nm s⁻¹ great); ^o number of likely metabolic reactions (range for 95% of drugs: 1–8); ^p predicted inhibition constants K_i^{pre} . K_i^{pre} was predicted from computed $\Delta\Delta G_{com}$ using the regression Equation B shown in Table 3; ^q human oral absorption (1 = low, 2 = medium, 3 = high); ^r percentage of human oral absorption in gastrointestinal tract (80% = high); * star in any column indicates that the property descriptor value of the compound falls outside the range of values for 95% of known drugs

3.5. Analysis of Novel COUs Analogs

The virtual library of novel COU analogs was designed based on structural information obtained from the active conformation of COUs. This information guided the selection

of suitable substituents at positions R₁, R₂, R₃, and R₄. To identify which substituents, contribute to the highest predicted potency against *NQO1*, we analyzed the frequency of occurrence of R₁, R₂, R₃, and R₄ among the top 63 PH4 hits. The results are presented as histograms in Figure 7. These histograms show that the substituent (F8) is the most frequent at

position R₁, with an occurrence frequency of 13. The fragments at position R₂ i.e., fragments (F10) and (F14) are the most repetitive and are followed by the substituent (F1, F3, F6 and F8) with occurrence frequencies of 7 and 6 respectively. As for the substituents at position R₃, the substituents with the highest occurrence frequency are the substituents (F8) and (F14), with frequencies of 16 and 12 respectively. Concerning the fragments at position R₄, the relative histogram reveals that the substituent (F3) is the most important with an occurrence rate of 14. Analysis of the frequencies of occurrence of substituents at R₁, R₂, R₃ and R₄ highlights recurring combinations among the most active analogues, including R₁-F8, R₂-F10, R₃-F8 and R₄-F3. These fragments appear to play a key role in enhancing biological activity and provide a solid basis for the design of new optimized derivatives.

The optimization of R₁ is particularly crucial, with fragment F8 being the most frequent (13 occurrences), suggesting that it significantly improves biological activity. In R₂, fragments F10 and F14 are predominant, followed by F1, F3, F6, and F8 (frequencies of 7 and 6 occurrences). In R₃, fragments F8 and F14 dominate (16 and 12 occurrences, respectively), indicating that this position plays a key role in affinity optimization. Finally, substituent F3 in R₄ is the most recurrent (14 occurrences), suggesting that it is essential for interaction with the target and influences selectivity. Statistical analysis of recurring substituents provides a rational basis for optimizing future COU analogs. These trends provide valuable information to guide the future design of analogs, thus optimizing their efficacy and selectivity.

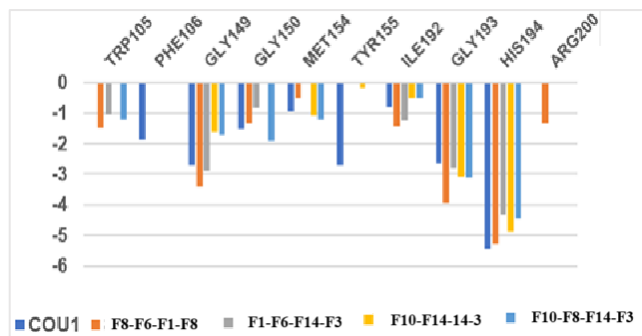


Figure 8. Molecular mechanics inter-molecular interaction energy *E_{int}* break-down to active site residue contributions in [kcal.mol⁻¹]: the best four novel designed COU analogs (the color coding refers to ligands given in the legend).

ADME profiles of designed

The pharmacokinetic profile of NQO1 inhibitors still requires further research. As presented in Table 9, the ADME properties of our novel analogs were previously analyzed using the QikProp program, based on Jorgensen's method. The fundamental principles of this approach have been described earlier. Our top-performing analogs are compared with commercially available drugs used for cancer treatment (Table 9).

4. Discussion

Beyond the robustness of the QSAR model, the analysis of interactions between COU and the active site residues revealed the key interactions responsible for COUs affinity with NQO1, including hydrogen bonds, van der Waals interactions, and hydrophobic contacts.

Additional insights into the interaction of ligands from the training set are provided by the NQO1-COU_x interaction energy diagram, which illustrates the individual energetic contributions of each active site residue in NQO1. The decomposition of interaction energy into individual contributions from NQO1's active site residues is crucial for identifying substituents that can enhance the binding affinity of COU analogs with NQO1 and, consequently, improve their inhibitory capacity. The individual contributions of interaction energies are categorized into three groups based on ligand activity levels within the training set: most active ligands, moderately active ligands and least active ligands. A comparative analysis of the interaction energy contributions across these groups (Figure 3) reveals that the binding affinity of the most active ligands is the strongest, highlighting key residues that play a significant role in ligand stabilization and potency.

The top 4 best hit fit from the PH4 result were evaluated with the QSAR complexation model, equation B from Table 3, in order to predict their inhibitory activity (pIC_{50}^{pre}): F10-F8-F14-F3: (pIC_{50}^{pre} = 10.69); F10-F14-F14-F3: (pIC_{50}^{pre} = 10.72); F1-F6-F14-F3: (pIC_{50}^{pre} = 11.73); F8-F6-F1-F8: (pIC_{50}^{pre} = 12.22).

5. Conclusion

Structural information from the crystallography of the NQO1-COU12 complex enabled the development of a reliable QSAR model for NQO1 inhibition by coumarin inhibitors, establishing a correlation between the calculated Gibbs free energies of complexation and the observed inhibitory potencies. Furthermore, a PH4 pharmacophore model was developed for these inhibitors using a training set of 22 coumarin derivatives. This model was validated with 6 coumarin derivatives for which their inhibitory. The analysis of interactions between NQO1 and coumarins within the enzyme's active site guided the design of an initial virtual combinatorial library of new coumarin analogues featuring various substitutions on the coumarin core. A refined selection, based on ADME-related descriptors and screening for alignment with the PH4 pharmacophore, allowed the identification of a subset of coumarins with potential oral bioavailability. This group, consisting of the 63 best virtual candidates, was then evaluated for predicted inhibitory activities using the QSAR complexation model, revealing analogues with estimated activities in the picomolar concentration range. The best designed COU analogues F10-F8-F14-F3: (IC_{50}^{pre} = 20.3 pM; pIC_{50}^{pre} =

10,69); F10-F14-F14-F3: ($IC_{50}^{pre} = 19.2 \text{ pM}$; $pIC_{50}^{pre} = 10,72$); F1-F6-F14-F3: ($IC_{50}^{pre} = 1.9 \text{ pM}$; $pIC_{50}^{pre} = 11,73$); F8-F6-F1-F8: ($IC_{50}^{pre} = 0.6 \text{ pM}$; $pIC_{50}^{pre} = 12,22$), Table 6, are recommended for synthesis and further evaluation of activity in NQO1 inhibition assays and could lead to the discovery of new potent anticancer agents, whether synthetic or natural, with good bioavailability.

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
ADME	Absorption, Distribution, Metabolism, and Excretion
COU	Coumarin Inhibitors
COUx	Coumarin Inhibitors of the Training Set
VCOUs	Coumarin Inhibitors of the Validation Set
$\Delta\Delta G_{com}$	Relative Complexation Gibbs Free Energy
GFE	Gibbs Free Energy
$\Delta\Delta G_{sol}$	Relative Solvation Gibbs Free Energy
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
H_{MM}	Enthalpy Component of Gibbs Free Energy
HOA	Human Oral Absorption
HYD	Hydrophobic
HYDA	Hydrophobic Aliphatic
K_i	Inhibitory Concentration
MM	Molecular Mechanics
MM-PB	Molecular Mechanics–poisson–boltzmann
NQO1	NAD(P)H Quinone Oxidoreductase 1
PDB	Protein Data Bank
PH4	Pharmacophore
QSAR	Quantitative Structure–activity Relationships
RMSD	Root-mean Square Deviation
SAR	Structure–activity Relationships
TS	Training Set
VS	Validation Set

Conflicts of Interest

The authors declare no conflicts of interest.

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