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Article

Flavonoids as Putative epi-modulators: Insights into their Binding Mode with BRD4 Bromodomain using Molecular Docking and Dynamics.

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Abstract: Flavonoids are widely recognized as natural polydrugs, given their anti-inflammatory, antioxidant, sedative and antineoplastic activities. Recently, different studies have shown that flavonoids have the potential to inhibit BET bromodomains. Previous reports suggest that flavonoids are putative inhibitors of the ZA channel due to their orientation and interactions with P86, V87, L92, L94 and N140. Herein, a comprehensive characterization of the binding mode of the biflavonoid amentoflavone and fisetin is discussed. To this end, both compounds were docked with BRD4 using four docking programs. Results were post-processed with protein-ligand interaction fingerprints. To gain further insights into the binding mode of the two natural products, docking results were further analyzed with molecular dynamics simulations. Results showed that amentoflavone makes numerous contacts in the ZA channel, as previously described for flavonoids and kinase inhibitors. It was also found that amentoflavone can potentially make contacts with non-canonical residues for BET inhibition. Most of these contacts were not observed with fisetin. Based on these results, amentoflavone was experimentally tested for BRD4 inhibition, showing activity in the micromolar range. This work may serve as basis for scaffold optimization and further characterization of flavonoids as BET inhibitors.

Keywords: docking, epigenetics, epi-informatics, molecular interactions, molecular dynamics, natural products, flavonoids.

1. Introduction

Epigenetics has arisen as the missing link in the biogenesis of disease. Histone modifications have significant effect on the fate of certain genes. Current research has been primarily focused on the writing and erasing mechanisms of the epigenome. There are plenty of examples of this in the literature [1], one of the most prominent being histone acetylation. Acetylation is regulated by two main systems: histone acetyl transferase (HATs) and histone deacetylases (HDACs) [2]. HDACs have been studied thoroughly by means of pharmacophore modelling [3], molecular docking [4] and molecular dynamics (MD) [5]. These efforts have contributed to the identification and development of two FDA approved HDAC inhibitors, the most notable being a natural product: romidepsin [6].

Readers are epi-enzymes whose function is to recognize certain modifications and their pattern on histones [7]. Therefore, reader enzymes are interesting molecular targets for a better understanding of epigenetics. Bromodomains are 120 residue proteins that were first discovered on the Brahma (brm) gene of Drosophilia genus [8]. Later it was confirmed as a common motif in most eukaryotic organisms. As of today, 62 isoforms have been identified and classified on eight families [9]. Family II, known as the bromodomain and extraterminal domain (BET) has been extensively studied as shown in Figure 1. This family includes BRD2, BRD3, BRD4, and BRDT isoforms, each with their respective first and second domains (BD1 and BD2). Figure 2 shows the active site of bromodomains,

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which is comprised by three main hotspots: WPF shelf; a region exclusive to BET bromodomains, it involves a hydrophobic region (residues 80 to 83); ZA channel, located between the Z and A loops (residues 85 through 96) often seen as a frontier region with mixed contacts (mainly hydrophobic). The third hotspot is the Ac-binding pocket, responsible for reading histones. This hotspot is defined by a "tandem checkpoint" made by N140 and Y97 [10].

Additionally, evidence of structural water molecules has been shown on a double bridge with ligands and Y97 [11]. Generally speaking, the role of water in binding is a dividing issue for drug design [12]. For example, structure-based design often ignores it or recognizes few instances of its importance [13]. Because of this, early approaches for ligand design followed a water displacement strategy [14]. Still, a slow but steady paradigm shift has come with increasing evidence of water-based stabilization in binding kinetics [15] and target selectivity [16]. One of the main problems in this approach is the increased difficulty of modelling of such phenomena, i.e., identifying "crucial waters" [17,18]. For bromodomains, recent studies have shown that the network of structural waters in the ZA channel plays a significant role on binding [19,20] and has served as a case study for the development of novel methods in the field [21].

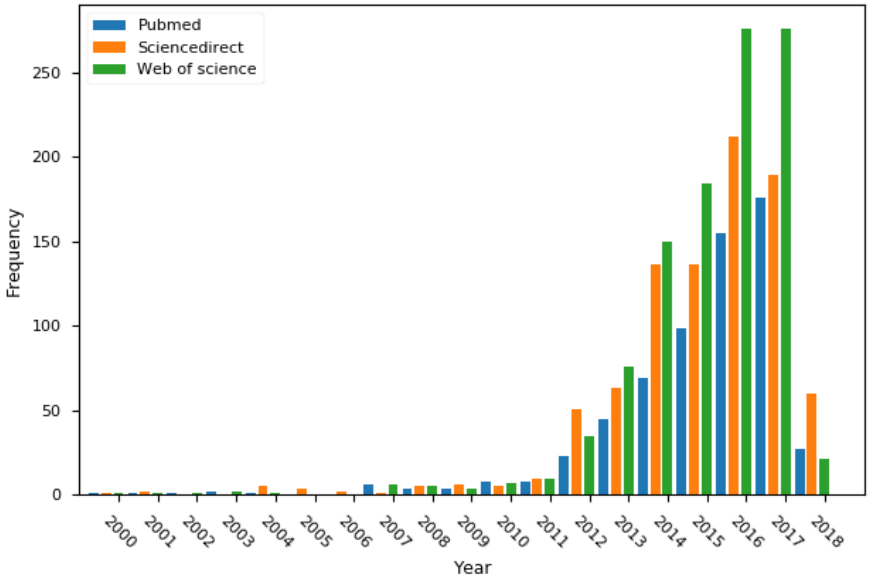

Figure 1. Frequency of "bromodomain" keyword in three major search engines during the past 18 years.

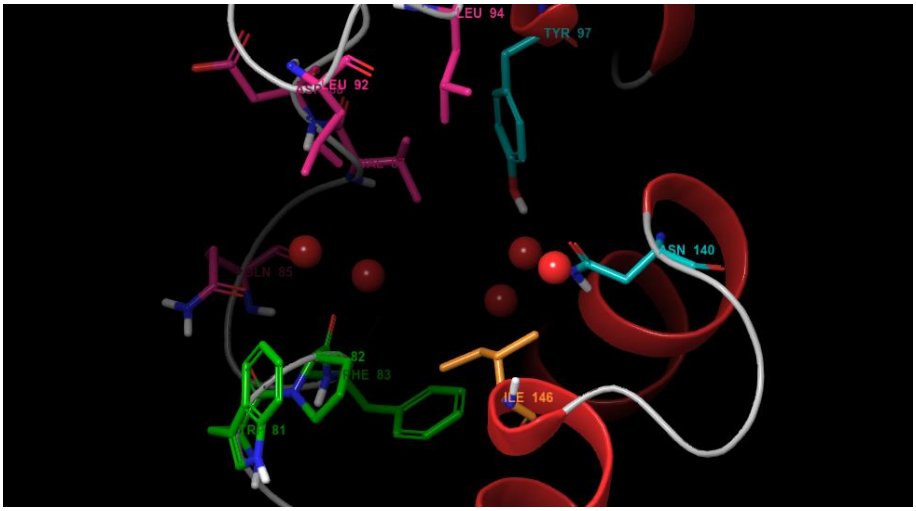

Figure 2. Binding pocket of BET bromomains. Main structural features are WPF shelf (green) ZA channel (pink), Ac-pocket (cyan), and the gatekeeper (orange). Red spheres represent structural waters, found on BET isoforms.

Bromodomain inhibition is currently on an impasse [22], as chemotypes are not diverse enough to make more robust models and approaches towards their pharmacology. Hence, current efforts are focused on the synthesis and identification of plausible and novel inhibitors [23]. As part of this effort, quinazolones have been proposed as novel inhibitors of BETs. An interesting property of these ligands is their selectivity towards BD2 [24]. Later it was found that some kinase inhibitors can bind to bromodomains [25], e.g. flavopiridol. Figure 3a illustrates quintessential BRD inhibitors. These results lead to the hypothesis of flavonoids as putative modulators of bromodomains, nonetheless this possibility has been explored only in recent studies [26].

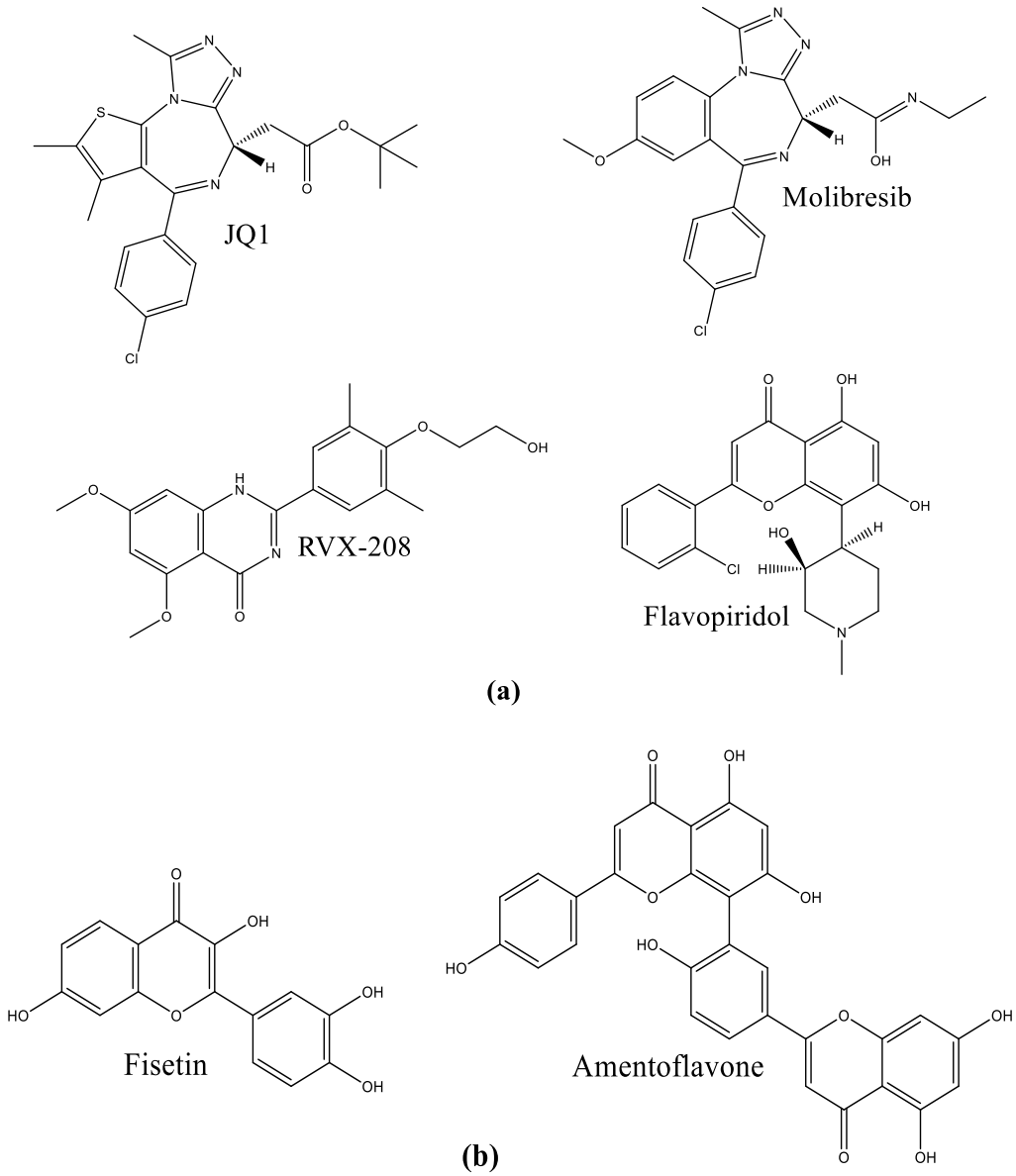

Figure 3. Chemical structures of: (a) reference ligands for BET inhibition; (b) Flavonoids studied in this work.

Flavonoids are one of the most well-known natural products, often regarded as major scaffolds in medicinal chemistry [27]. Flavonoids have shown antioxidant [28], anti-inflammatory [29] and sedative [30] effects in different studies. Moreover, flavonoid scaffolds present the outstanding

potential of being chemoprotective agents towards cancer [31]. In consequence, flavonoids are often seen as quintessential nutraceuticals e.g., the average intake of flavonoids in the United States is around 1 g/day [32]. Finally, it has been suggested that flavonoids may interact significantly with the epigenome, however as of today this has been limited to writing and eraser epi-enzymes [33].

Fisetin shown in Figure 3b, is a dietary flavonoid found on a broad array of vegetables such as strawberry, apple, grape, onion and cucumber [34] and is considered a health-promoting compound [35]. Studies have shown that fisetin is capable of blocking cell proliferation on many cancer lines [36]. One of the most interesting aspects of its pharmacology is its capacity to modulate NF-κβ [37]. Fisetin is capable to do this through the MAPK pathway and TNF-blocking, downregulating pro-inflammatory genes [38]. Of note, recent studies have shown the role of BRD4 on the recruitment of NF-κβ [39]. Thus, bromodomains have also been studied for their role on chronic disease like diabetes [40] and psoriasis [41]. Amentoflavone (see Figure 3b) is a biflavonoid produced by two apigenin units. It is commonly found on *Ginko biloba, Hypericum perforatum, Biophytum sensitivum* and *Nandina domestica* [42]. Like fisetin, amentoflavone has also been identified as a NF-κβ modulator [43], hence its capacity to reduce inflammation.

Computational methods are valuable approaches to solve chemical problems. Molecular docking for example, allows the simulation of protein-ligand binding. Despite its simplifications and limitations, docking has yielded significant results used for binding mode prediction [44]. Molecular dynamics, has gained increasing attention for elucidation of ligand binding and protein behavior [45].

Since amentoflavone and fisetin have been identified as putative ligands of BRD4 in two independent studies [46,47], a comprehensive characterization of the putative binding profile of both flavonoids with BRD4 is presented herein. The binding profile was carried out with consensus docking and molecular dynamics. Based on the computational results, amentoflavone was experimentally tested for activity as BRD4 inhibitor, showing activity in the micromolar range. These results further support the activity of flavonoids as putative epi-modulators.

2. Materials and Methods

2.1. Protein preparation

An ensemble of 14 structures for BET isoform BRD4 from the Protein Data Bank (PBD) was selected. Full details are presented in Table S1 of Supplementary Material. Selection criteria was based on their resolution (<1.8 Å) and R-value (<0.25). Additional criteria were the structural similarity between the co-crystal ligand and the flavonoid scaffold, and the ability of the ligand to form hydrogen bonds with the binding pocket. All protein-ligand complexes were prepared with the Quickprep module of MOE. Energy minimization was carried with the Amber 14:EHT force field. Complexes were visually inspected to ensure that key interactions were kept.

2.2. Molecular docking

Docking was carried out using four programs: Autodock Vina [48], LeDock [49], MOE (2018.01), and PLANTS [50]. The rationale to select these programs was their performance and different scoring functions for consensus (*vide infra*). Protein inputs were kept from the preparation step and validated with their respective native ligand. Details are provided in Table S2 in the Supplementary Material. Amentoflavone and fisetin were parametrized with Amber 14: EHT for MOE, and a charge reassignment for LeDock, Vina and PLANTS. The charge used for these programs was calculated by MOPAC 2016 [51] using PM6-D3H4X, as this correction has been shown to enhance docking performance [52]. The docking poses were post processed using protein-ligand interaction fingerprints (PLIF) as available in MOE. Docking poses were analyzed for clustering, based on the most common interactions found across the four programs.

2.3. Molecular dynamics

MD simulations were carried out using Desmond [53] for both BRD4 (see Supplementary Material, Figures S2-S3 and S6; Table S12) and BRD4-ligand complexes. The complex used was the top ranked pose from MOE with consensus interactions. Complexes were then submitted to the System Builder utility in Maestro to assign a buffered 10x10x10 Å orthorhombic box using TIP3P water model and OPLS_2005 forcefield. The system was neutralized and a 0.15 M concentration of NaCl was added. Further details can be found in Supplementary Material, Figure S1. Production time for MD was set at 100 ns. The simulation was repeated three times. Electrostatics were computed by the Particle Mesh Ewald algorithm with a 9 Å cutoff and constraints enforced by M-SHAKE. Integration was done every 1.2 fs, with recording interval set to 50 ps. Trajectories were then analyzed by the Simulation Interaction Diagram, Simulation Event Analysis, and Simulation Quality Analysis utilities in Maestro.

2.4. Experimental testing of amentoflavone

Amentoflavone was purchased from Sigma-Aldrich (St.Louis, MI) and tested for BRD4 tandem (BD1+BD2) binding, by means of Alpha Screening, using a H4 peptide (1-21) K5/8/12/16Ac. Experimental work was performed by Reaction Biology Corp. Providing 2 mg samples to obtain duplicate dose-response curves beginning at 100 μM concentration following a 3-fold dilution. Positive control for the test was JQ-1. IC_{50} values were obtained from the curves and the Hillslope for amentoflavone was calculated.

3. Results

3.1. Molecular docking

Table 1 summarizes the docking scores for amentoflavone and fisetin as computed with the four docking programs (the raw docking scores for each protein used are reported in the Supplementary Material, Tables S4-S11). Figure 4 shows the consensus PLIF found for both compounds.

Table 1. Summary statistics of docking scores for the programs used

Molecule	Summary stats*	Autodock VINA (kcal/mol)	LeDock (kcal/mol)	MOE (kcal/mol)	PLANTS
Amentoflavone	Min	-10.5	-7.9	-9.0	-102.1
	1Q	-9.5	-7.3	-7.9	-89.4
	Avg	-9.2	-7.0	-7.6	-86.9
	3Q	-9.0	-6.8	-7.2	-84.0
	Max	-8.2	-6.3	-6.4	-77.4
	SD	0.46	0.34	0.54	4.6
Fisetin	Min	-8.6	-6.0	-7.4	-79.6
	1Q	-8.2	-5.6	-6.5	-73.2
	Avg	-7.9	-5.4	-6.2	-71.0
	3Q	-7.7	-5.3	-6.0	-68.9
	Max	-7.1	-4.7	-5.6	-65.0
	SD	0.31	0.24	0.39	2.94

*Min, Minimum; 1Q, 1st quartile; Avg, average; 3Q, 3rd quartile; Max, Maximum and SD, Standard Deviation values.

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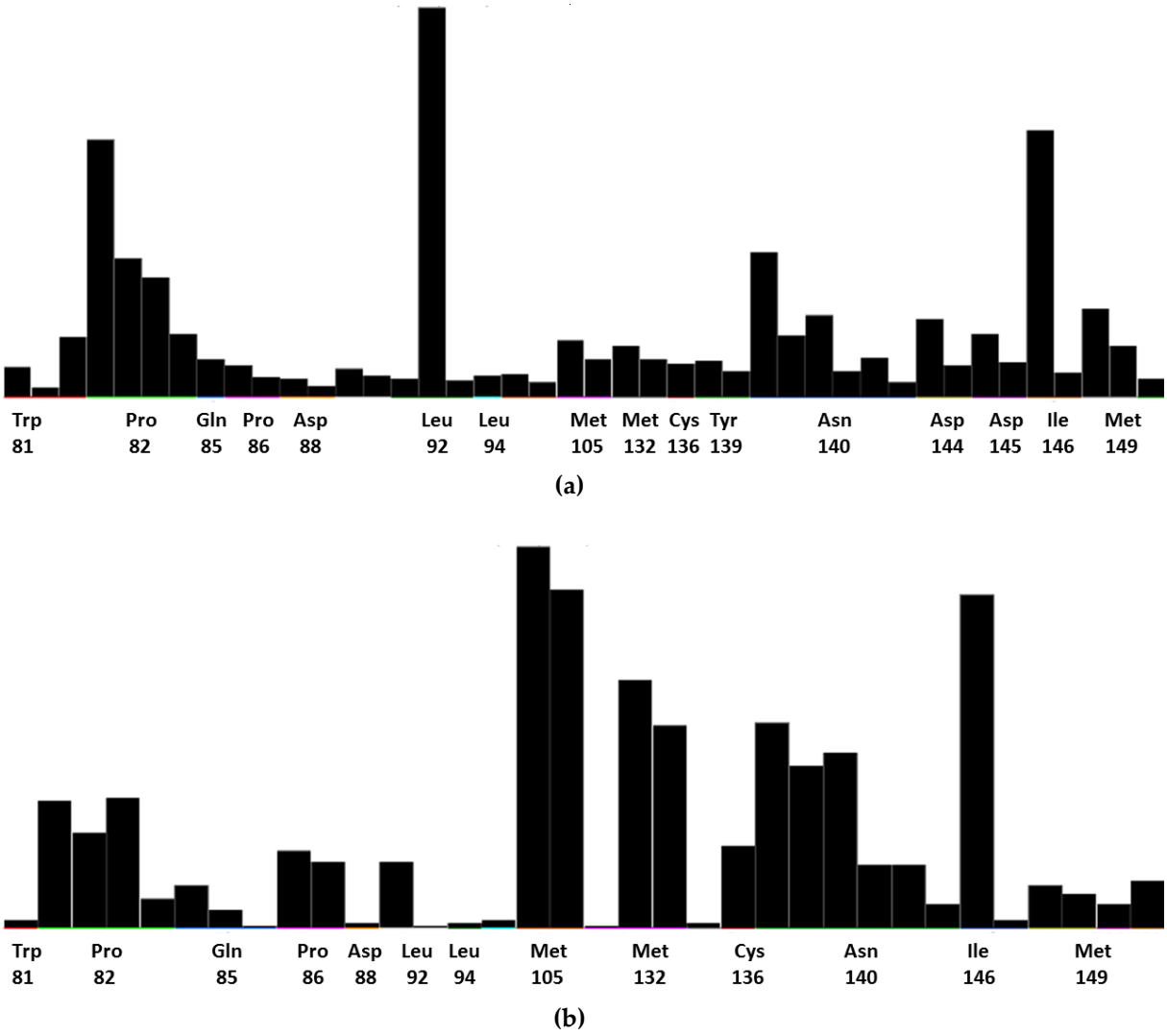

Figure 4. Consensus protein-ligand interaction fingerprint obtained from a consensus analysis of the docking with Autodock Vina, LeDock, MOE, and PLANTS. (a) Amentoflavone; (b) Fisetin.

3.2. Molecular dynamics

The overall quality of the MD simulations was measured with the corresponding utility in Maestro. Energy, potential energy, temperature, pressure and volume values were computed (results are shown in Figures S4-S5; Tables S13-S14, in the Supplementary Material). Once complex stability was assessed, RMSD values for backbone, Cα, sidechains and ligand were computed as seen in Figure 5a-b. This measure shows the global deviation of atoms to a reference status (frame 0); usually values below 5.0 Å can be considered as valid [54].

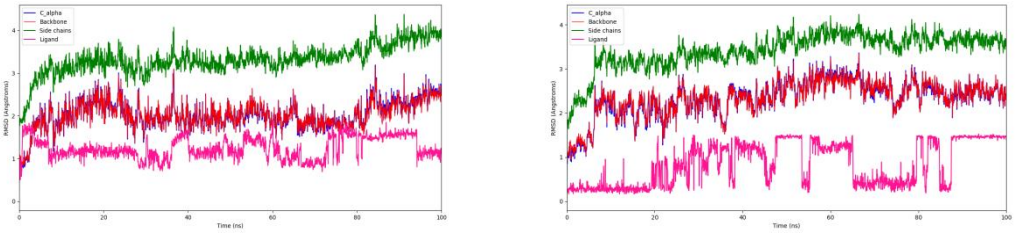

Figure 5. RMSD values for protein backbone, alpha carbons, side chain and ligand. (a) Amentoflavone; (b) Fisetin.

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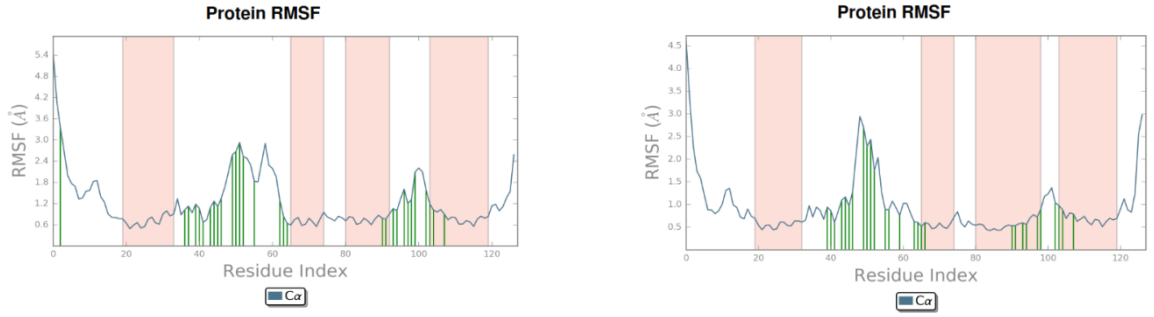
Root-mean square fluctuation (RMSF) was also calculated as seen in Figure 6a-b. These values show the general movement of each residue during the total simulation time. In this figure, the ligand contacts are shown as green lines matching the residue index, while the orange lines indicate protein secondary structures (helix, in this case). See Figure S6 in the Supplementary Material for further details.



(a) **(b)**

Figure 6. RMSF values based on alpha carbons, ligand contacts are presented in green and protein helix on orange. (a) Amentoflavone. (B) Fisetin.

Figures 7 and 8 show a protein-ligand contact analysis during MD simulations. Protein-ligand contacts can be interpreted as "dynamic PLIFs", showing the population of contacts during the simulation. Plots at the bottom of both figures represent the number of contacts and their density i.e., darker shade of orange indicates more than one contact on that frame. These also show the type of contact mapped to the structure of the ligand.

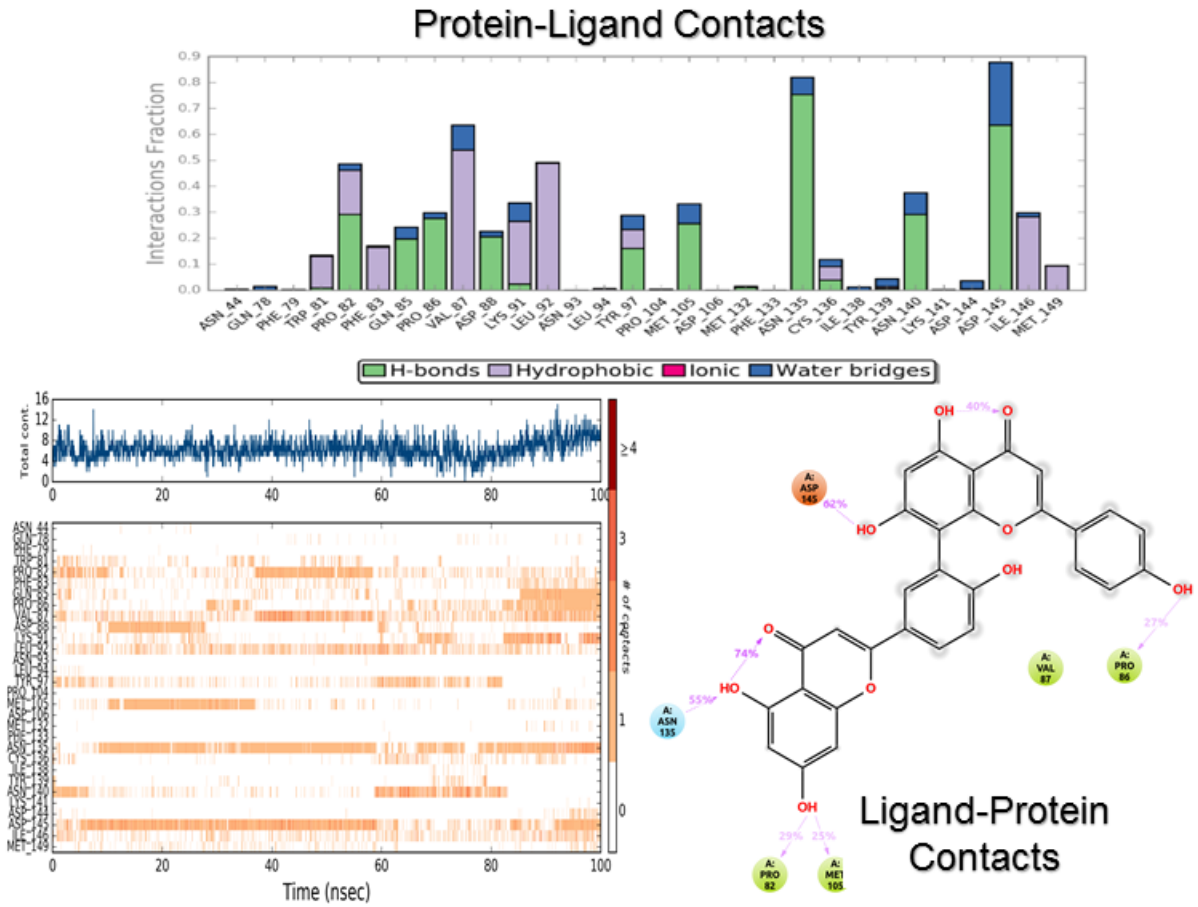


Figure 7. Protein-ligand contact analysis for amentoflavone during the MD simulation.

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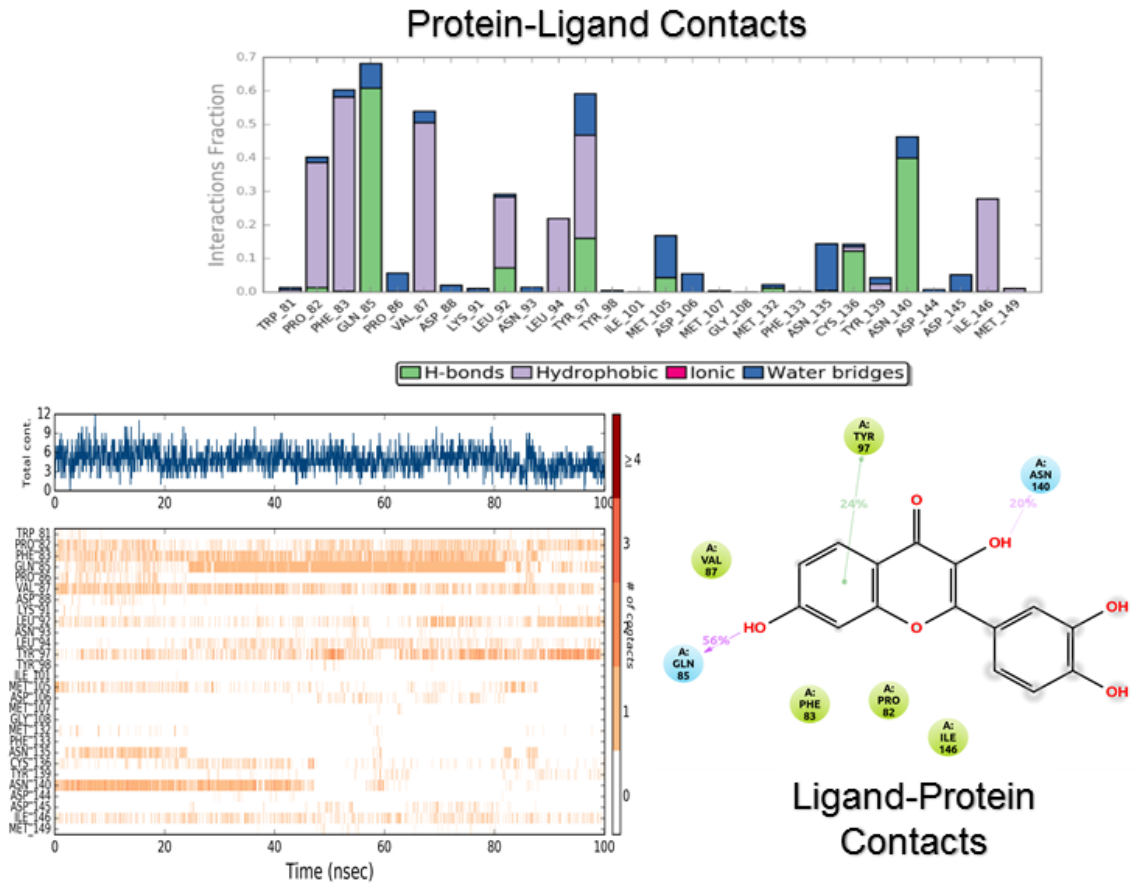
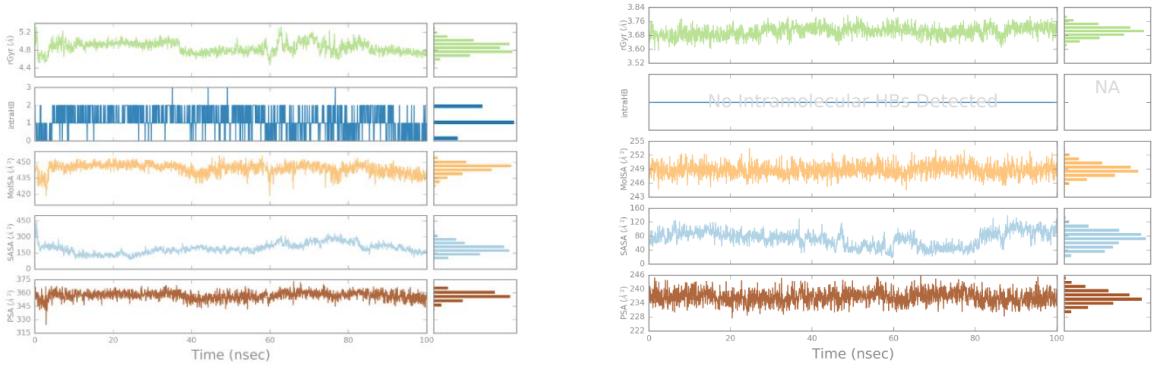


Figure 8. Protein-ligand contact analysis for fisetin during the MD simulation.

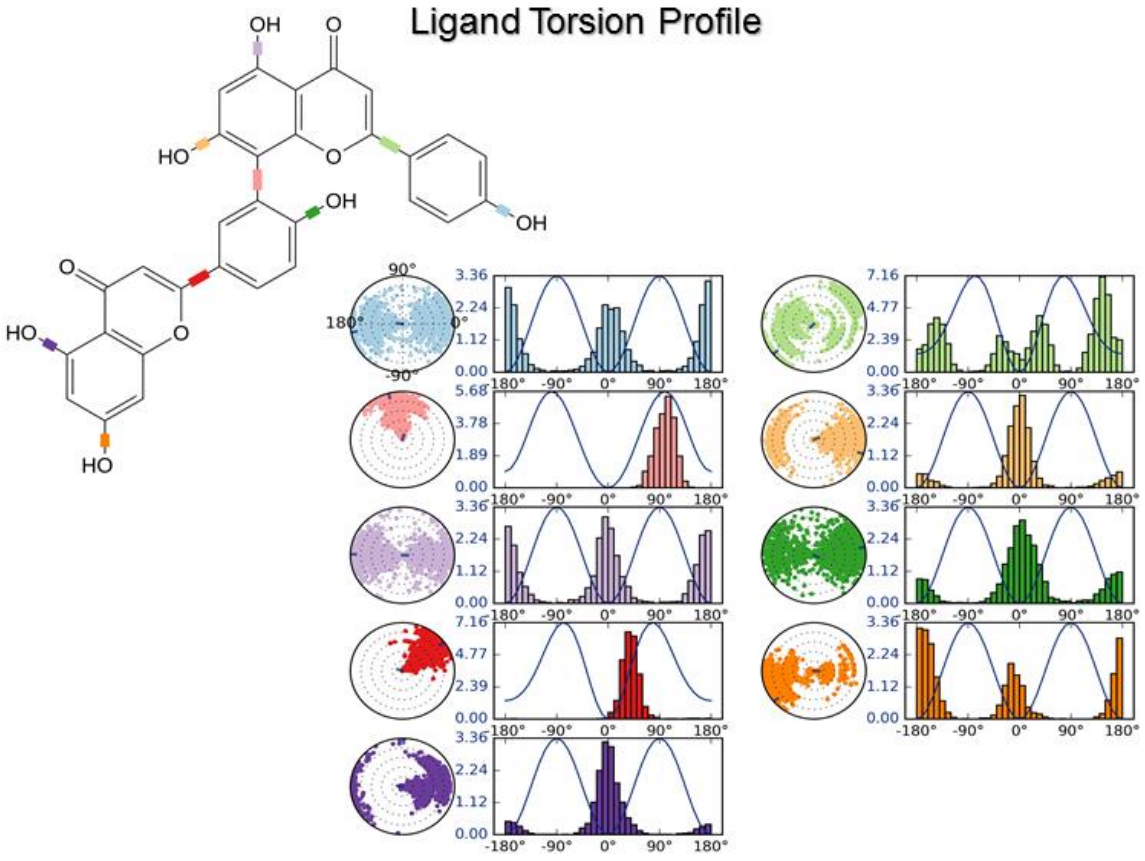
Figure 9a-b shows other ligand properties during the MD simulations. These include radius of gyration, intramolecular hydrogen bonding, VdW surface area, solvent accessible surface area and polar surface area. Of note, if a ligand is not capable of intramolecular hydrogen bonding this plot appears empty.



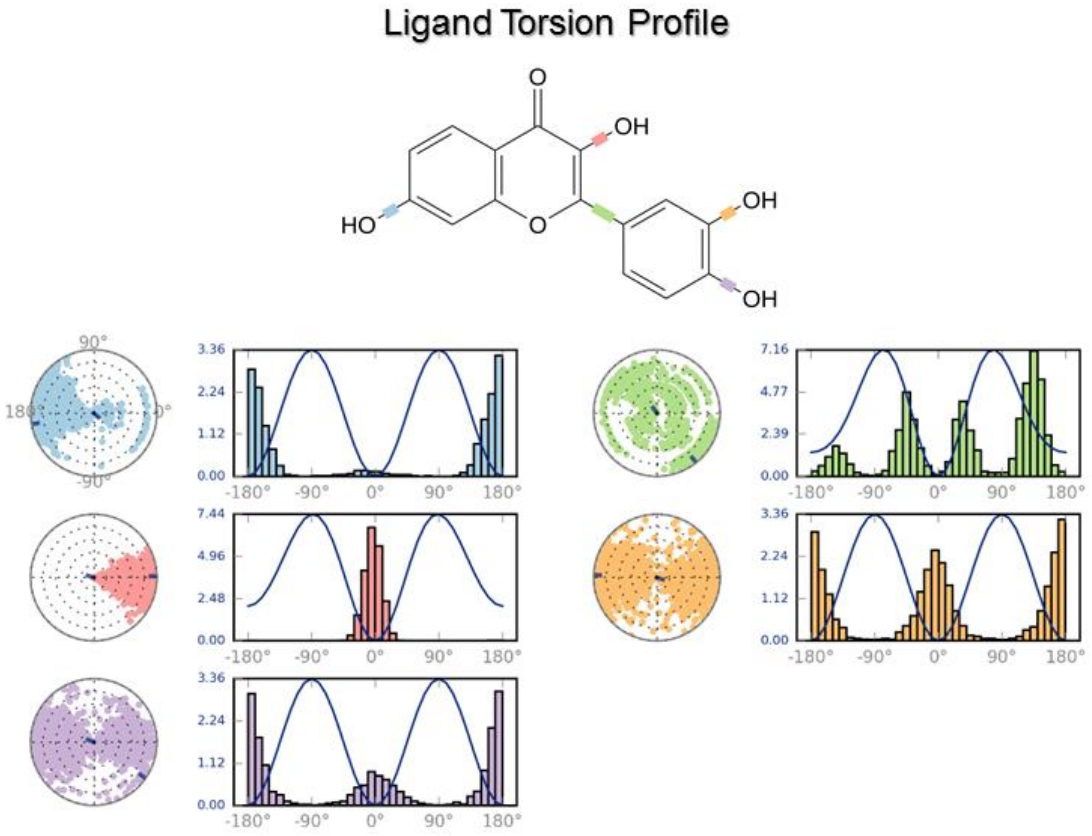
(a) **(b)**

Figure 9. Ligand properties during 100 ns simulations. (a) Amentoflavone. (b) Fisetin.

Figure 10a-b shows the energy values for dihedral angles (line plot), which account for torsional analysis. The histogram shows the density of probability of that torsion; while the dial on the left shows the rotation of that bond during the simulation (the beginning is marked by the center). Plots in Figure 10 allow determining if a given ligand undergoes torsional strain during binding.



(a)



(b)

Figure 10. Torsional analysis of ligand conformation during 100 ns. A) Amentoflavone. B) Fisetin. The color represents different rotatable bonds of the ligands. Values on the Y-axis are in kcal/mol.

3.3. Binding assay of amentoflavone

Table 2 summarizes the experimentally determined activity of amentoflavone†

Table 2. Values for IC and Hillslope of amentoflavone as obtained by Alpha Screening against BRD4 tandem.

	DATA 1	DATA 2
IC_{50} (**μM**)	36.1	30.4
HILLSLOPE	-2.5	-1.9

†As mentioned in the Methods section, experimental characterization was performed by Reaction Biology using Alpha Screening Assay.

4. Discussion

4.1. Molecular docking

Bromodomain inhibitors may be classified on two broad categories: Kac mimicking and non-mimicking, the former being the most prominent [55]. Flavonoids belong to this category as their carbonyl groups are their main anchor towards Asn140 and Tyr97 [46]. Nonetheless, as shown on Figure 4, these interactions are not as populated as it might be expected. Certainly, this may seem negative, as the Ac-pocket is the main anchor for bromodomain inhibition. Nonetheless the exclusion of molecules based solely on this criterion has been questioned [56].

Because of this, an integral approach based on ensemble docking and consensus scoring was conducted, as means to correctly assess the probability of a given interaction. Ensemble docking is a common technique used to account for protein flexibility [57] and has been applied successfully in several workflows [58]. Consensus scoring on the other hand, increases the rate of hit identification significantly [59]. However, the rate of success has a strong dependence on the selected programs for consensus. In consequence, a naïve choice leads to overestimation on weighted terms if similar parameters in scoring functions are used [60]. Hence, we selected the docking software based on searching algorithm capabilities and scoring function diversity. Briefly, the rationale for each selection is presented hereunder:

- Autodock VINA: It has a well stablished performance against several protein families, also its empirical scoring function has a significant correlation towards experimental values [61]. Finally, its hybrid search algorithm optimized by local search allows a better sampling of the free energy landscape [62].

- LeDock: Its search algorithm based on simulated annealing provides a significant clustering of poses. In addition, it has been implemented successfully in virtual screening campaigns for BET bromodomains [49].

- MOE: Its docking algorithm allows for induced fit search. Furthermore, its forcefield-based scoring function (using AMBER parameters with GB/VI solvation) considers the solvation contributions to ligand binding [63].

- PLANTS: It provides a notable sampling of side chain flexibility. Also, its searching algorithm (based on metaheuristics) and empirical scoring function have a well-stablished performance [64].

Additionally, a knowledge-based filtering was used to improve consensus results. This method takes advantage of PLIFs to identify trends in binding while selecting poses with “canonical” interactions [47]. Of note, the interactions of both flavonoids with Asn140 show a similar shift in MD

simulations which suggests a good sampling of our ensemble and notable performance the protocol presented herein.

Based on the docking scores, both amentoflavone and fisetin are comparable to those of currently known inhibitors (see Supplementary Material, Table S3). To the best of our knowledge, there are no studies or data showing the correlation of docking score and experimental binding energy of bromodomain inhibitors. While such analysis goes beyond the scope of this work, we provide reference values obtained from the literature (see Supplementary Material, Table S2).

Yet, docking scores calculated with LeDock are lower when compared to the scores computed with other programs. Nevertheless, this same trend was observed for reference inhibitors. This result is mostly due to the scoring function, as it has been shown that while accurate to identify correct binding poses, energy values assigned to them are often underestimated [65].

Additionally, the difference in score values for both compounds is significant. Roughly, these values suggest that amentoflavone could be three-fold more potent than fisetin. Arguably, this may be due to the bigger size of amentoflavone and higher number of hydroxyl groups giving it more anchors towards BRD4. Still, average scoring values rank them with a virtual IC_{50} around 1-5 μM, based on scaffold similarity and reference values.

Amentoflavone shows mainly hydrophobic contacts with the WPF shelf (Pro81) and the ZA channel (Leu 92). Fisetin on the other hand, shows more contacts with residues Met105 and Met132. Previous reports of docking indicate that flavonoids have a notable preference towards these residues [26], in the case of fisetin, affinity for Cys136 has also been observed [46]. This hints to flavonoids having a significant affinity towards different residues beyond the Ac-pocket, while their aromatic character gives them selectivity for the WPF shelf.

Interestingly, the consensus PLIF (Figure 4) shows that amentoflavone makes less contacts than fisetin. Moreover, the population of Asn140 bonding is significantly reduced for amentoflavone. In contrast, MD of both compounds show a similar interaction profile, whereas the fraction is higher for amentoflavone (0.9 vs 0.7). The Asn140 interaction fraction is similar for both flavonoids (around 0.4). Tyr97 on the other hand, makes a stronger and more lasting interaction with fisetin by means of pi stacking and hydrogen bonding. This may be due to the size of amentoflavone and its orientation in the protein cavity, evidenced by the contacts with "non-canonical" residues. An example of this is Asp145, a contact with amentoflavone with a rather small population. However, this contact has been identified as significant, as it provides ligand stabilization and water network interaction [66].

4.2. Molecular dynamics

As stated, MD simulations were conducted to contrast docking results and provide further insights in the binding mode of flavonoids. Based on protein RMSD values, BRD4 remains stable enough during the simulation with both flavonoids. Ligand RMSD on the other hand, shows higher deviations at times. This could suggest that both ligands undergo conformational changes during the simulation, i.e., two binding modes.

Therefore, torsional profiling plots assist on this interpretation (Figure 10). As these provide the spatial and energetic distribution of bond torsions during the simulation, showing both flavonoids are mostly strained in two main conformations. This could imply that fisetin changes its conformation more quickly than amentoflavone, due to rotation on its catechol ring. However, this observation differs from previous report [37], which suggests that fisetin keeps a restrained conformation when bound to BRD4. The main reason for this may be related to the use of different forcefields (OPLS_2005 vs OPLS3). On this matter, it is noteworthy that other ligand properties (Figure 9) show similar trend values for fisetin as in said study.

Amentoflavone on the other hand, keeps a restrained conformation on its shared phenol ring. This behavior can be related to atropisomerism features, present on the biflavonoid. Based on these results it can be hypothesized that amentoflavone activity on BETs is mediated by atropisomerism. Of course, stability studies of amentoflavone and its atropisomers are due to confirm this hypothesis. However, such techniques and focus are beyond the aims of this work. Suffice to say, even though such phenomenon may be common in biflavonoids its recognition in medicinal chemistry is often overlooked [67]. Moreover, interest on atropisomerism is recently increasing [68]. Thus, we believe that knowledge of this feature could improve novel ligand designs, giving a paradigm shift mostly needed for these targets.

RMSF plots (Figure 6) also show that the protein-ligand complexes remain consistently stable and the main secondary structure are four α-helices, which confirms a correct sampling of the system. These plots also show that main contacts in both flavonoids are with the ZA channel, with high fluctuations on these residues during the simulation. Interestingly, when these protein-ligand contacts are analyzed, different interaction profiles arise for both flavonoids.

Amentoflavone clearly makes more contacts with the ZA channel as the MD simulation goes on. Also, its presence in the cavity makes a significant impact on the secondary structure of the protein, increasing the helix portion of this region (Figure S6 in the Supporting Material). As stated above, this may be related to the bigger size of the structure. However, based on the "contact-mixture" this can be also related to the strained conformation of the molecule allowing a more favorable angle towards hydrogen bonding and the hydrophobic interactions.

Again, contact with Asp145 is remarkable. In this case being the most populated in the MD with amentoflavone. Also, the presence of water bridges with this residue proved significant, a feature recently observed by other groups [66]. Furthermore, this residue is present only on the BRD4 BD1, providing specific contact with histone H3 via hydrogen bonding, an interaction not present with inhibitors such as JQ-1 [69]. This would suggest that amentoflavone can be selective for the first domain of BRD4. This is noteworthy, considering molecular similarity towards RVX-208 would suggest selectivity for BRD4-BD2.

4.3. Experimental evaluation

Based on the results of molecular docking and dynamics, it was decided to acquire a sample and experimentally test amentoflavone as a BRD4 inhibitor. Fisetin was not considered for testing due to a previous report of quercetin showing an IC_{50} of 38 μM [70]. With this value as reference, our efforts focused on the biflavonoid scaffold. It is very positive that amentoflavone showed significant binding for BRD4, with an IC_{50} on the micromolar range. Indeed, being more potent when compared to a flavonoid monomer (38 vs 30 μM). Additionally, its Hillslope value could indicate that amentoflavone is indeed selective for one domain of BRD4. Still, more testing is due, i.e., binding to separate domains of BRD4.

Plus, this experimental confirmation provides further evidence of flavonoids as general chemoprotective agents. One of the main concerns about the use of flavonoids as nutraceutics is their putative toxicity, as fisetin and other flavonoid monomers inhibit DNA topoisomerases [71] and actin polymerization [36]. Biflavonoids on the other hand, do not present this feature, however have been reported as potentially mutagenic [72]. Nonetheless, such negative effects are only present at concentrations between 100 and 250 μM [73,74]. As such, based on the IC_{50} of both quercetin and amentoflavone, flavonoids have a significant potential as epi-nutraceutics.

Summarizing, the work presented serves as a remarkable proof-of-concept for both; flavonoids as epi-modulators and the computational methods used hereunder. Putting the results together amentoflavone shows the characteristic contacts previously reported for flavonoid reports i.e.,

strong contacts with the ZA region plus novel predicted interactions with Asp145 and the water network. Biological tests supported the hypothesis of binding and plausible selectivity.

Despite the fact that flavonoids have small room for optimization and break Lipinski´s rule of five, their true potential is as chemoprotective agents. As previously mentioned, this finding further advances the field of nutriepigenomics. Moreover, it is remarkable that these natural products provide pharmacophoric templates for novel inhibitors of an epigenetic target.

5. Conclusions

Amentoflavone is a natural product with several associated biological effects. Its ability to block NF-κβ is the key towards its anti-inflammatory potential. BETs have been identified as NF-κβ promoters, with JQ-1 being highly effective on psoriasis models. Previously, a similar effect has been reported for amentoflavone. Based on these results and other reports, we conducted a binding characterization of this ligand and compared to fisetin, another flavonoid with reports of putative activity. We present a consensus docking methodology which allows binding characterization and hit selection. Certainly, such approach is impractical for large virtual screening campaigns. However, based on the performance and results presented, it provides a powerful tool for pose selection as supported by MD results.

Simulations conducted herein indicated that amentoflavone can make numerous contacts in the ZA channel, as previously described for flavonoids and kinase inhibitors. It was also determined that amentoflavone can potentially make contacts with "non-canonical" residues for BET inhibition e.g., Met105, Asn135, Cys136 and Asp145. Most of these contacts were not observed with fisetin (except for Cys136). Based on analysis of torsional values, it is plausible that this behavior is due to the atropisomerism present in the molecule. As a first step towards testing this hypothesis, *in vitro* inhibition of BRD4 was evaluated. Experimental evaluation showed that amentoflavone is indeed active in the micromolar range, with plausible selectivity against one domain in the BRD4 tandem.

Perspectives of this work include the experimental testing of fisetin and contrast its result with the molecular modeling predictions. Additionally, for amentoflavone specific tests for BD1 and BD2 are due, to confirm its selectivity. Finally, we consider these results while preliminary, offer a new paradigm for inhibitor design and characteristics for novel modulation of BETs.

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References

1. Cabaye, A.; Nguyen, K. T.; Liu, L.; Pande, V.; Schapira, M. Structural diversity of the epigenetics pocketome. *Proteins Struct. Funct. Bioinforma.* **2015**, *83*, 1316–1326, doi:10.1002/prot.24830.

2. Eberharter, A.; Becker, P. B. Histone acetylation: A switch between repressive and permissive chromatin. Second in review on chromatin dynamics. *EMBO Rep.* **2002**, *3*, 224–229, doi:10.1093/embo-reports/kvf053.

3. Kalyaanamoorthy, S.; Chen, Y. P. P. Energy based pharmacophore mapping of HDAC inhibitors against class i HDAC enzymes. *Biochim. Biophys. Acta - Proteins Proteomics* **2013**, *1834*, 317–328, doi:10.1016/j.bbapap.2012.08.009.

4. Ortore, G.; Colo, F. Di; Martinelli, A. Docking of Hydroxamic Acids into HDAC1 and HDAC8: A Rationalization of Activity Trends and Selectivities. *J. Chem. Inf. Model.* **2009**, *49*, 2774–2785, doi:10.1021/ci900288e.

5. Hassanzadeh, M.; Bagherzadeh, K.; Amanlou, M. A comparative study based on docking and molecular dynamics simulations over HDAC-tubulin dual inhibitors. *J. Mol. Graph. Model.* **2016**, *70*, 170–180, doi:10.1016/j.jmgm.2016.10.007.

6. VanderMolen, K. M.; McCulloch, W.; Pearce, C. J.; Oberlies, N. H. Romidepsin (Istodax, NSC 630176, FR901228, FK228, depsipeptide): a natural product recently approved for cutaneous T-cell lymphoma. *J. Antibiot. (Tokyo).* **2011**, *64*, 525–31, doi:10.1038/ja.2011.35.

7. Arrowsmith, C. H.; Bountra, C.; Fish, P. V.; Lee, K.; Schapira, M. Epigenetic protein families: a new frontier for drug discovery. *Nat. Rev. Drug Discov.* **2012**, *11*, 384–400, doi:10.1038/nrd3674.

8. Berkovits, B. D.; Wolgemuth, D. J. The Role of the Double Bromodomain-Containing BET Genes During Mammalian Spermatogenesis. *Curr. Top. Dev. Biol.* **2013**, *102*, 293–326, doi:10.1016/B978-0-12-416024-8.00011-8.

9. Hewings, D. S.; Rooney, T. P. C.; Jennings, L. E.; Hay, D. A.; Schofield, C. J.; Brennan, P. E.; Knapp, S.; Conway, S. J. Progress in the Development and Application of Small Molecule Inhibitors of Bromodomain–Acetyl-lysine Interactions. *J. Med. Chem.* **2012**, *55*, 9393–9413, doi:10.1021/jm300915b.

10. Ferri, E.; Petosa, C.; McKenna, C. E. Bromodomains: Structure, function and pharmacology of inhibition. *Biochem. Pharmacol.* **2016**, *106*, 1–18, doi:10.1016/j.bcp.2015.12.005.

11. Brand, M.; Measures, A. M.; Wilson, B. G.; Cortopassi, W. A.; Alexander, R.; Höss, M.; Hewings, D. S.; Rooney, T. P. C.; Paton, R. S.; Conway, S. J. Small Molecule Inhibitors of Bromodomain–Acetyl-lysine Interactions. *ACS Chem. Biol.* **2015**, *10*, 22–39, doi:10.1021/cb500996u.

12. Ladbury, J. E. Just add water! The effect of water on the specificity of protein-ligand binding sites and its potential application to drug design. *Chem. Biol.* **1996**, *3*, 973–980, doi:10.1016/S1074-5521(96)90164-7.

13. Plumridge, T. H.; Waigh, R. D. Water structure theory and some implications for drug design. *J. Pharm. Pharmacol.* **2002**, *54*, 1155–79, doi:10.1211/002235702320402008.

14. Crawford, T. D.; Tsui, V.; Flynn, E. M.; Wang, S.; Taylor, A. M.; Côté, A.; Audia, J. E.; Beresini, M. H.; Burdick, D. J.; Cummings, R.; Dakin, L. A.; Duplessis, M.; Good, A. C.; Hewitt, M. C.; Huang, H. R.; Jayaram, H.; Kiefer, J. R.; Jiang, Y.; Murray, J.; Nasveschuk, C. G.; Pardo, E.; Poy, F.; Romero, F. A.; Tang, Y.; Wang, J.; Xu, Z.; Zawadzke, L. E.; Zhu, X.; Albrecht, B. K.; Magnuson, S. R.; Bellon, S.; Cochran, A. G. Diving into the Water: Inducible Binding Conformations for BRD4, TAF1(2), BRD9, and CECR2 Bromodomains. *J. Med. Chem.* **2016**, *59*, 5391–5402, doi:10.1021/acs.jmedchem.6b00264.

15. Pan, A. C.; Borhani, D. W.; Dror, R. O.; Shaw, D. E. Molecular determinants of drug-receptor binding kinetics. *Drug Discov. Today* **2013**, *18*, 667–673, doi:10.1016/j.drudis.2013.02.007.

16. Huggins, D. J.; Sherman, W.; Tidor, B. Rational approaches to improving selectivity in drug design. *J. Med. Chem.* **2012**, *55*, 1424–1444, doi:10.1021/jm2010332.

17. Ross, G. A.; Morris, G. M.; Biggin, P. C. Rapid and accurate prediction and scoring of water molecules in protein binding sites. *PLoS One* **2012**, *7*, e32036, doi:10.1371/journal.pone.0032036.

18. García-Sosa, A. T.; Firth-Clark, S.; Mancera, R. L. Including tightly-bound water molecules in de novo drug design. exemplification through the in silico generation of poly(ADP-ribose)polymerase ligands. *J. Chem. Inf. Model.* **2005**, *45*, 624–633, doi:10.1021/ci049694b.

19. Shadrick, W. R.; Slavish, P. J.; Chai, S. C.; Waddell, B.; Connelly, M.; Low, J. A.; Tallant, C.; Young, B. M.; Bharatham, N.; Knapp, S.; Boyd, V. A.; Morfouace, M.; Roussel, M. F.; Chen, T.; Lee, R. E.; Kiplin Guy, R.; Shelat, A. A.; Potter, P. M. Exploiting a water network to achieve enthalpy-driven, bromodomain-selective BET inhibitors. *Bioorganic Med. Chem.* **2018**, *26*, 25–36, doi:10.1016/j.bmc.2017.10.042.

20. Bharatham, N.; Slavish, P. J.; Young, B. M.; Shelat, A. A. The role of ZA channel water-mediated interactions in the design of bromodomain-selective BET inhibitors. *J. Mol. Graph. Model.* **2018**, *81*, 197–210, doi:10.1016/j.jmgm.2018.03.005.

21. Geist, L.; Mayer, M.; Cockcroft, X. L.; Wolkerstorfer, B.; Kessler, D.; Engelhardt, H.; McConnell, D. B.; Konrat, R. Direct NMR Probing of Hydration Shells of Protein Ligand Interfaces and Its Application to Drug Design. *J. Med. Chem.* **2017**, *60*, 8708–8715, doi:10.1021/acs.jmedchem.7b00845.

22. Prieto-Martínez, F. D.; Fernandez-de Gortari, E.; Méndez-Lucio, O.; Medina-Franco, J. L. A

chemical space odyssey of inhibitors of histone deacetylases and bromodomains. *RSC Adv.* **2016**, *6*, 56225–56239, doi:10.1039/C6RA07224K.

23. Galdeano, C.; Ciulli, A. Selectivity on-target of bromodomain chemical probes by structure-guided medicinal chemistry and chemical biology. *Future Med. Chem.* **2016**, *8*, 1655–1680, doi:10.4155/fmc-2016-0059.

24. Kharenko, O. A.; Gesner, E. M.; Patel, R. G.; Norek, K.; White, A.; Fontano, E.; Suto, R. K.; Young, P. R.; McLure, K. G.; Hansen, H. C. RVX-297- a novel BD2 selective inhibitor of BET bromodomains. *Biochem. Biophys. Res. Commun.* **2016**, *477*, 62–67, doi:10.1016/j.bbrc.2016.06.021.

25. Ember, S. W. J.; Zhu, J. Y.; Olesen, S. H.; Martin, M. P.; Becker, A.; Berndt, N.; Georg, G. I.; Schonbrunn, E. Acetyl-lysine binding site of bromodomain-containing protein 4 (BRD4) interacts with diverse kinase inhibitors. *ACS Chem. Biol.* **2014**, *9*, 1160–1171, doi:10.1021/cb500072z.

26. Dhananjayan, K. Molecular Docking Study Characterization of Rare Flavonoids at the Nac-Binding Site of the First Bromodomain of BRD4 (BRD4 BD1). *J. Cancer Res.* **2015**, *2015*, 1–15, doi:10.1155/2015/762716.

27. Singh, M.; Kaur, M.; Silakari, O. Flavones: An important scaffold for medicinal chemistry. *Eur. J. Med. Chem.* **2014**, *84*, 206–239, doi:10.1016/j.ejmech.2014.07.013.

28. Perry, N. S. L.; Bollen, C.; Perry, E. K.; Ballard, C. Salvia for dementia therapy: Review of pharmacological activity and pilot tolerability clinical trial. *Pharmacol. Biochem. Behav.* 2003, *75*, 651–659.

29. Hwang, S. L.; Shih, P. H.; Yen, G. C. Citrus Flavonoids and Effects in Dementia and Age-Related Cognitive Decline. In *Diet and Nutrition in Dementia and Cognitive Decline*; Elsevier, 2014; pp. 869–878 ISBN 9780124079397.

30. Fernández, S. P.; Wasowski, C.; Paladini, A. C.; Marder, M. Synergistic interaction between hesperidin, a natural flavonoid, and diazepam. *Eur. J. Pharmacol.* **2005**, *512*, 189–198, doi:10.1016/j.ejphar.2005.02.039.

31. Thilakarathna, W. W.; Langille, M. G.; Rupasinghe, H. V. Polyphenol-based prebiotics and synbiotics: potential for cancer chemoprevention. *Curr. Opin. Food Sci.* **2018**, *20*, 51–57, doi:10.1016/j.cofs.2018.02.011.

32. Khan, N.; Syed, D. N.; Ahmad, N.; Mukhtar, H. Fisetin: A Dietary Antioxidant for Health Promotion. *Antioxid. Redox Signal.* **2013**, *19*, 151–162, doi:10.1089/ars.2012.4901.

33. Vasantha Rupasinghe, H. P.; Nair, S. V. G.; Robinson, R. A. *Chemopreventive properties of fruit phenolic compounds and their possible mode of actions*; 1st ed.; Elsevier B.V., 2014; Vol. 42; ISBN 9780444632814.

34. Sung, B.; Pandey, M. K.; Aggarwal, B. B. Fisetin , an Inhibitor of Cyclin-Dependent Kinase 6 , Down- Regulates Nuclear Factor-kB-Regulated Cell Proliferation , Antiapoptotic and Metastatic Gene Products through the Suppression of TAK-1 and Receptor-Interacting Protein- Regulated IkB Kinase. *Mol. Pharmacol.* **2007**, *71*, 1703–1714, doi:10.1124/mol.107.034512.cannot.

35. Syed, D. N.; Adhami, V. M.; Khan, N.; Khan, M. I.; Mukhtar, H. Exploring the molecular targets of dietary flavonoid fisetin in cancer. *Semin. Cancer Biol.* **2016**, *40–41*, 130–140, doi:10.1016/j.semcancer.2016.04.003.

36. Sundarraj, K.; Raghunath, A.; Perumal, E. A review on the chemotherapeutic potential of fisetin: In vitro evidences. *Biomed. Pharmacother.* **2018**, *97*, 928–940, doi:10.1016/j.biopha.2017.10.164.

37. Kashyap, D.; Sharma, A.; Sak, K.; Tuli, H. S.; Buttar, H. S.; Bishayee, A. Fisetin: A bioactive phytochemical with potential for cancer prevention and pharmacotherapy. *Life Sci.* **2018**, *194*, 75–87, doi:10.1016/j.lfs.2017.12.005.

38. Rengarajan, T.; Yaacob, N. S. The flavonoid fisetin as an anticancer agent targeting the growth signaling pathways. *Eur. J. Pharmacol.* **2016**, *789*, 8–16, doi:10.1016/j.ejphar.2016.07.001.

39. Nadeem, A.; Al-Harbi, N. O.; Al-Harbi, M. M.; El-Sherbeeny, A. M.; Ahmad, S. F.; Siddiqui, N.; Ansari, M. A.; Zoheir, K. M. A.; Attia, S. M.; Al-Hosaini, K. A.; Al-Sharary, S. D. Imiquimod-induced psoriasis-like skin inflammation is suppressed by BET bromodomain inhibitor in mice through RORC/IL-17A pathway modulation. *Pharmacol. Res.* **2015**, *99*, 248–257, doi:10.1016/j.phrs.2015.06.001.

40. Coletta, D. K. Genetic and Epigenetics of Type 2 Diabetes. In *Pathobiology of Human Disease*; Elsevier, 2014; pp. 467–476 ISBN 9780123864567.

41. Mele, D. A.; Salmeron, A.; Ghosh, S.; Huang, H.-R.; Bryant, B. M.; Lora, J. M. BET bromodomain inhibition suppresses T H 17-mediated pathology. *J. Exp. Med.* **2013**, *210*, 2181–2190, doi:10.1084/jem.20130376.

42. Burkard, M.; Leischner, C.; Lauer, U. M.; Busch, C.; Venturelli, S.; Frank, J. Dietary flavonoids and modulation of natural killer cells: implications in malignant and viral diseases. *J. Nutr. Biochem.* **2017**, *46*, 1–12, doi:10.1016/j.jnutbio.2017.01.006.

43. Catarino, M. D.; Talhi, O.; Rabahi, A.; Silva, A. M. S.; Cardoso, S. M. The Antiinflammatory Potential of Flavonoids. In *Studies in Natural Products Chemistry*; Elsevier B.V., 2016; Vol. 48, pp. 65–99 ISBN 9780444636027.

44. Onawole, A. T.; Sulaiman, K. O.; Adegoke, R. O.; Kolapo, T. U. Identification of potential inhibitors against the Zika virus using consensus scoring. *J. Mol. Graph. Model.* **2017**, *73*, 54–61, doi:10.1016/j.jmgm.2017.01.018.

45. Śledź, P.; Caflisch, A. Protein structure-based drug design: from docking to molecular dynamics. *Curr. Opin. Struct. Biol.* **2018**, *48*, 93–102, doi:10.1016/j.sbi.2017.10.010.

46. Raj, U.; Kumar, H.; Varadwaj, P. K. Molecular docking and dynamics simulation study of flavonoids as BET bromodomain inhibitors. *J. Biomol. Struct. Dyn.* **2016**, *1102*, 1–12, doi:10.1080/07391102.2016.1217276.

47. Prieto-Martínez, F. D.; Medina-Franco, J. L. Charting the Bromodomain BRD4: Towards the Identification of Novel Inhibitors with Molecular Similarity and Receptor Mapping. *Lett. Drug Des. Discov.* **2018**, *15*, 1–10, doi:10.2174/1570180814666171121145731.

48. Trott, O.; Olson, A. J. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **2009**, *31*, NA-NA, doi:10.1002/jcc.21334.

49. Unzue, A.; Zhao, H.; Lolli, G.; Dong, J.; Zhu, J.; Zechner, M.; Dolbois, A.; Caflisch, A.; Nevado, C. The "gatekeeper" Residue Influences the Mode of Binding of Acetyl Indoles to Bromodomains. *J. Med. Chem.* **2016**, *59*, 3087–3097, doi:10.1021/acs.jmedchem.5b01757.

50. Korb, O.; Stützle, T.; Exner, T. E. Empirical scoring functions for advanced Protein-Ligand docking with PLANTS. *J. Chem. Inf. Model.* **2009**, *49*, 84–96, doi:10.1021/ci800298z.

51. Stewart, J. J. P. Application of the PM6 method to modeling the solid state. *J. Mol. Model.* **2008**, *14*, 499–535, doi:10.1007/s00894-008-0299-7.

52. Hostaš, J.; Řezáč, J.; Hobza, P. On the performance of the semiempirical quantum mechanical PM6 and PM7 methods for noncovalent interactions. *Chem. Phys. Lett.* **2013**, *568–569*, 161–166, doi:10.1016/j.cplett.2013.02.069.

53. Bowers, K.; Chow, E.; Xu, H.; Dror, R.; Eastwood, M.; Gregersen, B.; Klepeis, J.; Kolossvary, I.; Moraes, M.; Sacerdoti, F.; Salmon, J.; Shan, Y.; Shaw, D. Scalable Algorithms for Molecular Dynamics Simulations on Commodity Clusters. In *ACM/IEEE SC 2006 Conference (SC'06)*; 2006.

54. Sargsyan, K.; Grauffel, C.; Lim, C. How Molecular Size Impacts RMSD Applications in Molecular Dynamics Simulations. *J. Chem. Theory Comput.* **2017**, *13*, 1518–1524, doi:10.1021/acs.jctc.7b00028.

55. Romero, F. A.; Taylor, A. M.; Crawford, T. D.; Tsui, V.; Côté, A.; Magnuson, S. Disrupting Acetyl-Lysine Recognition: Progress in the Development of Bromodomain Inhibitors. *J. Med. Chem.* **2016**, *59*, 1271–1298, doi:10.1021/acs.jmedchem.5b01514.

56. Ran, T.; Zhang, Z.; Liu, K.; Lu, Y.; Li, H.; Xu, J.; Xiong, X.; Zhang, Y.; Xu, A.; Lu, S.; Liu, H.; Lu, T.; Chen, Y. Insight into the key interactions of bromodomain inhibitors based on molecular docking, interaction fingerprinting, molecular dynamics and binding free energy calculation. *Mol. Biosyst.* **2015**, *11*, 1295–1304, doi:10.1039/c4mb00723a.

57. Korb, O.; Olsson, T. S. G.; Bowden, S. J.; Hall, R. J.; Verdonk, M. L.; Liebeschuetz, J. W.; Cole, J. C. Potential and Limitations of Ensemble Docking. *J. Chem. Inf. Model.* **2012**, 1262–1274, doi:10.1021/ci2005934.

58. Evangelista, W.; Weir, R. L.; Ellingson, S. R.; Harris, J. B.; Kapoor, K.; Smith, J. C.; Baudry, J. Ensemble-based docking: From hit discovery to metabolism and toxicity predictions. *Bioorganic Med. Chem.* **2016**, *24*, 4928–4935, doi:10.1016/j.bmc.2016.07.064.

59. Tuccinardi, T.; Poli, G.; Romboli, V.; Giordano, A.; Martinelli, A. Extensive consensus docking evaluation for ligand pose prediction and virtual screening studies. *J. Chem. Inf. Model.* **2014**, *54*, 2980–2986, doi:10.1021/ci500424n.

60. Kitchen, D. B.; Decornez, H.; Furr, J. R.; Bajorath, J. Docking and scoring in virtual screening for drug discovery: methods and applications. *Nat Rev Drug Discov* **2004**, *3*, 935–949, doi:10.1038/nrd1549.

61. Palestro, P. H.; Gavernet, L.; Estiu, G. L.; Bruno Blanch, L. E. Docking Applied to the Prediction of the Affinity of Compounds to P-Glycoprotein. *Biomed Res. Int.* **2014**, *2014*, 1–10, doi:10.1155/2014/358425.

62. Ferreira, L.; dos Santos, R.; Oliva, G.; Andricopulo, A. Molecular Docking and Structure-Based Drug Design Strategies. *Molecules* **2015**, *20*, 13384–13421, doi:10.3390/molecules200713384.

63. Naïm, M.; Bhat, S.; Rankin, K. N.; Dennis, S.; Chowdhury, S. F.; Siddiqi, I.; Drabik, P.; Sulea, T.; Bayly, C. I.; Jakalian, A.; Purisima, E. O. Solvated Interaction Energy (SIE) for scoring protein-ligand binding affinities. 1. Exploring the parameter space. *J. Chem. Inf. Model.* **2007**, *47*, 122–133, doi:10.1021/ci600406v.

64. Richter, L.; de Graaf, C.; Sieghart, W.; Varagic, Z.; Mörzinger, M.; de Esch, I. J. P.; Ecker, G. F.; Ernst, M. Diazepam-bound GABAA receptor models identify new benzodiazepine binding-site ligands. *Nat. Chem. Biol.* **2012**, *8*, 455–464, doi:10.1038/nchembio.917.

65. Wang, Z.; Sun, H.; Yao, X.; Li, D.; Xu, L.; Li, Y.; Tian, S.; Hou, T. Comprehensive evaluation of ten docking programs on a diverse set of protein-ligand complexes: the prediction accuracy of sampling power and scoring power. *Phys. Chem. Chem. Phys.* **2016**, *18*, 12964–12975, doi:10.1039/c6cp01555g.

66. Hoffer, L.; Voitovich, Y. V.; Raux, B.; Carrasco, K.; Muller, C.; Fedorov, A. Y.; Derviaux, C.; Amouric, A.; Betzi, S.; Horvath, D.; Varnek, A.; Collette, Y.; Combes, S.; Roche, P.; Morelli, X. Integrated Strategy for Lead Optimization Based on Fragment Growing: The Diversity-Oriented-Target-Focused-Synthesis Approach. *J. Med. Chem.* **2018**, acs.jmedchem.8b00653, doi:10.1021/acs.jmedchem.8b00653.

67. Waterman, M. J.; Nugraha, A. S.; Hendra, R.; Ball, G. E.; Robinson, S. A.; Keller, P. A. Antarctic Moss Biflavonoids Show High Antioxidant and Ultraviolet-Screening Activity. *J.*

Nat. Prod. **2017**, *80*, 2224–2231, doi:10.1021/acs.jnatprod.7b00085.

68. Glunz, P. W. Recent encounters with atropisomerism in drug discovery. *Bioorganic Med. Chem. Lett.* **2018**, *28*, 53–60, doi:10.1016/j.bmcl.2017.11.050.

69. Jung, M.; Philpott, M.; Müller, S.; Schulze, J.; Badock, V.; Eberspächer, U.; Moosmayer, D.; Bader, B.; Schmees, N.; Fernández-Montalván, A.; Haendler, B. Affinity map of bromodomain protein 4 (BRD4) interactions with the histone H4 tail and the small molecule inhibitor JQ1. *J. Biol. Chem.* **2014**, *289*, 9304–9319, doi:10.1074/jbc.M113.523019.

70. Andrews, F. H.; Singh, A. R.; Joshi, S.; Smith, C. A.; Morales, G. A.; Garlich, J. R.; Durden, D. L.; Kutateladze, T. G. Dual-activity PI3K–BRD4 inhibitor for the orthogonal inhibition of MYC to block tumor growth and metastasis. *Proc. Natl. Acad. Sci.* **2017**, *114*, E1072–E1080, doi:10.1073/pnas.1613091114.

71. Scotti, L.; Bezerra Mendonca, F. J.; Ribeiro, F. F.; Tavares, J. F.; da Silva, M. S.; Barbosa Filho, J. M.; Scotti, M. T. Natural Product Inhibitors of Topoisomerases: Review and Docking Study. *Curr. Protein Pept. Sci.* **2018**, *19*, 275–291, doi:10.2174/1389203718666170111114442.

72. Cardoso, C. R. P.; Cólus, I. M. de S.; Bernardi, C. C.; Sannomiya, M.; Vilegas, W.; Varanda, E. A. Mutagenic activity promoted by amentoflavone and methanolic extract of Byrsonima crassa Niedenzu. *Toxicology* **2006**, *225*, 55–63, doi:10.1016/j.tox.2006.05.003.

73. Lee, E.-J.; Shin, S.-Y.; Lee, J.-Y.; Lee, S.-J.; Kim, J.-K.; Yoon, D.-Y.; Woo, E.-R.; Kim, Y.-M. Cytotoxic Activities of Amentoflavone against Human Breast and Cervical Cancers are Mediated by Increasing of PTEN Expression Levels due to Peroxisome Proliferator-Activated Receptor γ Activation. *Bull. Korean Chem. Soc.* **2012**, *33*, 2219–2223, doi:10.5012/bkcs.2012.33.7.2219.

74. Grynberg, N. F.; Carvalho, M. G.; Velandia, J. R.; Oliveira, M. C.; Moreira, I. C.; Braz- Filho, R.; Echevarria, A. DNA topoisomerase inhibitors: Biflavonoids from Ouratea species. *Brazilian J. Med. Biol. Res.* **2002**, *35*, 819–822, doi:10.1590/S0100-879X2002000700009.