

In silico screening for CDK5 inhibitors leads to SAR analysis of flavonoids, three diverse inhibitor scaffolds and fisetin demonstrating potent *in vitro* glioblastoma effects

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ARTICLE INFO

Keywords:

CDK5
Fisetin
Flavonoids
Glioblastoma
Inhibitors
Kinase

ABSTRACT

There is an urgent need for more effective treatment protocols for glioblastoma (GBM), the most common malignant brain tumour in adults. Kinase inhibitors have demonstrated significant potential, with cyclin-dependant kinase 5 (CDK5) recognised as a promising target. Here, we present an *in silico* led approach towards the discovery of CDK5 inhibitors with anti-GBM potential. Active/decoy benchmarking computations considered the available CDK5–ligand complex crystal structures, to design and optimize a virtual screening protocol that involved 3D pharmacophore pre-filtering of compounds and Glide docking calculations. Screening of the ZINC15 biogenic and Analyticon Discovery natural product databases led to the identification of six low micromolar CDK5/p35 flavonoid inhibitors, with fisetin ($IC_{50} = 0.90 \mu M$) and apigenin ($IC_{50} = 1.19 \mu M$) validated as the most potent. Additionally, three diverse chemical scaffolds with xanthone, aristolactam and β -carboline cores were identified as hit compounds for further optimization. Structure activity relationship (SAR) analysis of sixteen flavonoids tested in the CDK5/p35 *in vitro* binding assays unravelled structural features governing the observed potencies, while selectivity screening against five homologous kinases revealed cirsiolol as the most selective flavonoid for CDK5 ($IC_{50} = 4.73 \mu M$). Meanwhile, fisetin demonstrated the most potent effects on cell viability ($IC_{50}s \sim 20\text{--}40 \mu M$ at 72 h) against three GBM cell lines (U87-MG, T98G and U251-MG), in a generally time- and concentration-dependent manner.

1. Introduction

Glioblastoma (GBM) is a highly aggressive brain tumour with the highest incidence rates found in developed countries such as the United States (US), and accounts for the largest proportion (~50%) of newly diagnosed malignant brain and other CNS tumours [1]. The majority of GBM cases occur in those over 40 years of age, with the incidence increasing with age and peaking at 75–84 years [2]. Survival data for GBM have shown that in most cases the median survival is <15 months [2]. The current standard of care is the Stupp protocol and consists of maximal safe surgical resection (where possible) followed by concurrent radiotherapy with temozolomide (TMZ) chemotherapy, and subsequent adjuvant TMZ for six cycles [3]. TMZ acts as a DNA methylating agent, however, sensitivity and resistance is a key issue, mainly due to some patients having a O⁶-methylguanine-DNA methyltransferase (MGMT)

positive status facilitating DNA repair. Additionally, glioma stem cell (GSC) populations are inherently resistant to both chemotherapy and radiotherapy, and further sustain tumour recurrence [4]. Other approaches such as tumour treating fields (TTFields) are used to prolong survival [5], however, there is a dire and urgent need for new chemotherapeutic approaches with greater efficacy than is currently seen.

Kinase inhibitors have been a productive source of new anti-cancer therapies [6], with the aberrant activity of Cyclin-Dependent Kinases (CDKs) frequently linked with cancer [7], including brain cancer [8]. Because of this, CDK inhibitor discovery for novel GBM therapeutics is actively being explored [8–11]. CDKs play key roles in cell cycle control and transcription regulation. Among the CDK family of kinases, CDK5 is involved in brain development functions such as neuronal cell survival, neuronal differentiation and synaptic plasticity [12]. Dysregulated CDK5 activity contributes to GBM proliferation and metastasis [13,14],

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<https://doi.org/10.1016/j.molstruc.2026.147607>

Received 17 July 2026; Received in revised form 12 September 2026; Accepted 13 September 2026

Available online 15 September 2026

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with CDK5 expression in glioma ~6-fold greater compared to normal brain tissue[15]. CDK5 inhibition in GBM has shown promise in a number of areas. Prevention of GSC self-renewal has been observed [16], while concomitant use of a CDK5 inhibitor with TMZ or irradiation, caused DNA damage and better survival in xenograft mouse models [17]. Recently, type-II inhibitors targeting CDK5 were discovered that demonstrated anti-GBM potential in cellular models [10], with type-II inhibition stabilizing the inactive state providing the possibility of achieving longer duration of action and potential for greater clinical efficacy [18,19]. Despite these promising observations, it is unlikely that single kinase inhibition is sufficient for GBM treatment, particularly due to treatment resistance and tumour recurrence. GBM is defined by considerable intratumoral heterogeneity, such that a single tumour mass contains multiple genetically and phenotypically distinct subpopulations of cells, with differing sensitivities to treatments [20]. Multiple kinase inhibitors (MKIs) can have better therapeutic outcomes through synergistic effects and having potential to overcome resistance [10,21,22].

Natural products and their analogues have made a major contribution to pharmacotherapy over the years, including for cancer [23–25]. In this study, candidate ATP-binding site (type-I) CDK5 inhibitors were predicted through *in silico* screening of natural product-based compounds from the ZINC15 biogenic [26] and Analyticon Discovery natural product databases, and then validated by *in vitro* CDK5/p35 binding assays. An initial benchmarking study considered solved CDK5-ligand crystal structure complexes from the protein data bank (PDB, www.rcsb.org) [27], 3D pharmacophore modelling and different Glide docking protocols to optimize a virtual screening approach. The benchmarking used known potent CDK5 inhibitors as actives and their generated decoys, with subsequent statistical analysis of performance for recognition of actives. The final optimized protocol was then used for virtual screening of the databases, with twenty-eight candidate inhibitors selected for *in vitro* CDK5/p35 binding assay validation, that included sixteen flavonoids and twelve diverse chemical scaffolds. IC₅₀ values were determined for the most potent compounds. The most potent flavonoids were then tested for their anti-GBM potential at the cellular level using three established cell-lines (U87-MG, T98G, U251-MG).

2. Results & discussion

2.1. *In silico* screening benchmarking studies

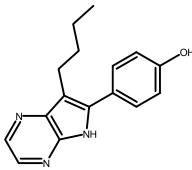
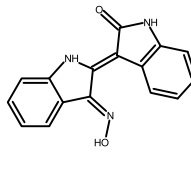
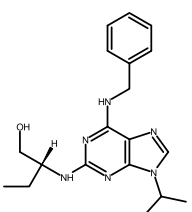
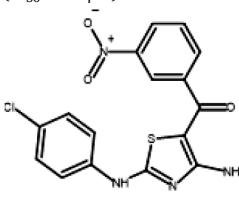
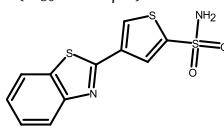
2.1.1. Native and cross-docking studies

As an initial test of Glide docking accuracy for the CDK5 ATP-binding site, re-docking of native ligands (Table 1) from five CDK5 – ligand crystallographic complexes (PDB IDs: 3O0G, 1UNG, 1UNH, 1UNL, and 4AU8) as well as cross docking into the other proteins was performed, and the ligand RMSDs determined. The RMSD values were generated from Glide-SP (no constraints) docking poses, with a RMSD value < 2.0 Å generally considered a good value for docking poses compared to their crystallographic conformations. As shown in Table 2, docking of the five native ligands into their respective proteins showed excellent reproducibility of the crystallographic binding modes (RMSDs of 0.124–1.153 Å). For cross-docking, there was no real stand-out protein, with all PDBs having three ligand RMSDs < 2 Å. 1UNL could be proposed as best performing, with four of the five ligands docked to this protein conformation having RMSD values < 2.5 Å.

The volume of the binding site can influence binding predictions. Studies have shown a more open pocket can lead to better performance in active/decoy ligand docking [31]. Binding site volumes of the ATP-binding sites in each of the five PDBs was calculated using Schrödinger's SiteMap [32]. The results are reported in Table S1, revealing that the volumes vary between ~205–260 Å³. The protein structure with the smallest binding site is 1UNG, which therefore could have a reduced number of docked compounds and the structures with

Table 1

PDB codes of the CDK5 protein-ligand crystal structures used for benchmarking studies. The PDB resolutions (Å), native ligand chemical structures and CDK5 binding assay IC₅₀s [28–30] are also shown.

1UNG [28] Resolution: 2.30 Å Ligand: Aloisine A (IC₅₀ = 0.16 μM) 	1UNH [28] Resolution: 2.35 Å Ligand: Indirubin-3'-oxime (IC₅₀ = 0.10 μM) 
1UNL [28] Resolution: 2.20 Å Ligand: R-Roscovitine (IC₅₀ = 0.16 μM) 	3O0G [29] Resolution: 1.95 Å Ligand: {4-amino-2-[(4-chlorophenyl)amino]-1,3-thiazol-5-yl}(3-nitrophenyl)methanone (IC₅₀ = 2.0 μM) 
4AU8 [30] Resolution: 1.90 Å Ligand: 4-(1,3-benzothiazol-2-yl)thiophene-2-sulfonamide (IC₅₀ = 0.55 μM) 	

largest binding sites were 1UNL (260.3 Å³) and 3O0G (256.2 Å³). However, variation in the volumes is generally relatively small, so volume may not have a large impact on the return of active ligands across the different proteins in docking calculations.

2.1.2. Benchmarking docking studies

Glide-SP docking was next carried out using an active(50 ligands)/decoy(2900) benchmarking set against the five PDB's to statistically analyze performance against each PDB for recognition of actives. The statistical results from these calculations are shown in Table 3. Notably, the PDB: 3O0G out-performed all other PDBs across every metric. The Enrichment Factor (EF) represents recovery for different percentages of the total number of ligands tested. EF for the top 1%, 5% and 10% of the database was analysed, although the top 1% (top-30 ligands in this case) had the highest importance for us and was prioritised for assessing accuracy. EF values assess method capability for selecting actives as opposed to random selection; an EF of 10, for example, indicates 10 times more likely to select an active. The Diverse Enrichment Factor (DEF) additionally considers structural diversity in the recovery of actives. The EF-1% and DEF-1% values for 3O0G were 20 and 19, respectively. One limitation of EF and DEF metrics is that they do not consider the early and late recognition of actives, however, the Boltzmann-enhanced discrimination of receiver operating characteristic (BEDROC, range 0–1), modified EF (EF'), and modified DEF (DEF') do, with values for 3O0G of 0.45, 43 (EF'–1%) and 43 (DEF'–1%), respectively. These values were all considerably higher than observed for the other PDBs. Taking the 3O0G protein forward, the performance of Glide-SP versus -XP was compared. The results also shown in Table 3 revealed Glide-SP as the most accurate for this system.

Table 2

RMSD (Å) for Glide-SP native and cross-docking calculations of five PDB ligands. Values below RMSD of 2.0 Å are coloured green, values between 2.0 Å and 3.0 Å are coloured amber, and values greater than 3.0 Å are coloured red.

	Protein					
	PDB	3O0G	1UNG	1UNH	1UNL	4AU8
Ligand	3O0G	0.326	5.015	6.517	6.634	5.022
	1UNG	1.309	1.153	5.778	1.596	4.466
	1UNH	6.024	0.721	0.146	0.409	0.468
	1UNL	0.893	1.610	1.061	0.602	1.733
	4AU8	7.380	7.290	1.791	2.456	0.124

Table 3

Enrichment statistical results for actives recovery in the benchmarking studies using Glide-SP docking against the five different CDK5 PDBs: 3O0G, 1UNG, 1UNH, 1UNL and 4AU8.^a

Enrichment statistics ^a		4AU8	1UNG	1UNH	1UNL	3O0G ^b
EF	1%	14	12	9.8	16	20 (12)
	5%	5.6	7.2	4	6.8	8.8 (7.2)
	10%	3.2	4.4	2.8	4.6	6.2 (4.4)
EF'	1%	25	19	17	30	43 (19)
	5%	9.9	10	5.7	11	15 (10)
	10%	6	7.4	4.2	7.3	10 (7.4)
DEF	1%	14	12	9.7	16	19 (12)
	5%	5.5	7.2	3.9	6.7	8.8 (7.2)
	10%	3.2	4.4	2.8	4.6	6.2 (4.4)
DEF'	1%	24	18	17	29	43 (18)
	5%	9.8	10	5.7	11	15 (10)
	10%	5.9	7.4	4.2	7.2	10 (7.4)
BEDROC	$\alpha=20.0$	0.28	0.33	0.21	0.33	0.45 (0.33)
Number of ranked actives		47	49	49	49	49 (50)

^a Best results are highlighted in **bold italics** for each parameter. ^b Glide-XP results are given in parentheses for comparison.

Towards further improving Glide-SP docking results for recovery of actives using PDB: 3O0G, two further protocols were designed and tested using the active/decoy ligand set. **Protocol 1** (results of which were described above) involved Glide-SP docking (without constraints),

protocol 2 involved Glide-SP docking with hydrogen bond constraints for the ATP-binding site hinge region (Fig. 1A), with a requirement to match at least 2 of the 3 defined hydrogen bond constraints. **Protocol 3** incorporated **protocol 2** but with the addition of a pre-screening 3D pharmacophore filter, with a criterion to match at least 3 of the 4 pharmacophore features. The pharmacophore shown in Fig. 1B superimposed on to the native ligand of PDB: 1UNG contained a hydrogen bond acceptor (HBA), hydrogen bond donor (HBD) and 2 aromatic groups (R). Hinge region interactions are a crucial aspect for the binding of inhibitors to mimic the adenine moiety of the ATP which binds to the hinge region. More specifically for CDK5, hydrogen bond interactions with the Glu81 backbone O and Cys83 backbone O and NH were employed. Planarity of ligands around the hinge region is complementary to the ATP-binding cavity for CDK5 (and other kinases), therefore planarity of the ligand poses was also focused on during the design and selection process [29]. The pharmacophore ensures that compounds with poor planarity and/or compounds which don't have at least 3 of the 4 model features were filtered out, due to a presumed poorer inhibitory effect.

The enrichment calculator statistical performance of the three protocols was compared and is shown in Table 4. **Protocols 2 and 3** generally showed significant improvement in parameters over **protocol 1**. EF-1% and DEF-1% were improved for both **protocol 2** and **3** returning the same values for each protocol (24 and 23, respectively). For EF'-1% and DEF'-1%, **protocol 3** (49 and 48, respectively) outperformed **protocol 2** (40 and 40, respectively), taking into the account the early recognition of actives. BEDROC was similar for **protocols 1–3** (0.45–0.47). A decrease in number of actives recovered was observed for

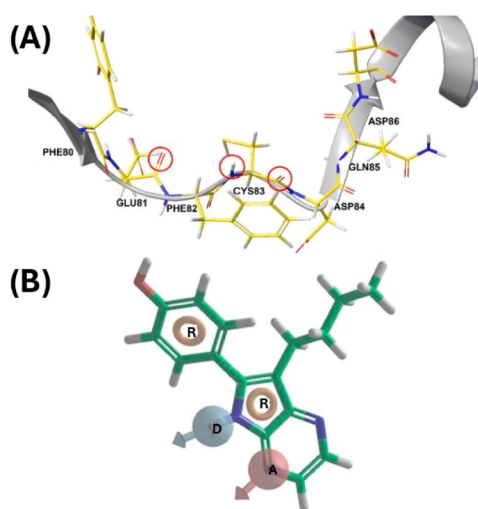


Fig. 1. (A) Hydrogen bond constraints (circled in red) used during docking, involving hinge region Glu81 backbone O and Cys83 backbone NH and O. (B) Selected 4 feature 3D-pharmacophore model used as a pre-screening filter; A: hydrogen bond acceptor, D: hydrogen bond donor, R: Aromatic groups. Pharmacophore was superimposed onto the native ligand from PDB: 1UNG (Aloisine A).

Table 4

Enrichment statistical results for actives recovery in the benchmarking set using three different docking protocols and PDB: 3O0G. **Protocol 1**: Glide-SP docking without hydrogen bond constraints. **Protocol 2**: Glide-SP docking with at least 2 of 3 hinge region hydrogen bonds constraints with the backbone Glu81 O and Cys83 NH and O. **Protocol 3**: Pre-screening 3D-pharmacophore filtering to match at least 3 of 4 features (Fig. 1B) combined with **protocol 2**.

Enrichment statistics ^a		Protocol 1	Protocol 2	Protocol 3
EF	1%	20	24	24
	5%	8.8	9.6	8.8
	10%	6.2	6.2	5.4
EF'	1%	43	40	49
	5%	15	17	17
	10%	10	11	9.7
DEF	1%	19	23	23
	5%	8.8	9.5	8.8
	10%	6.2	6.2	5.4
DEF'	1%	43	40	48
	5%	15	17	17
	10%	10	11	9.7
BEDROC	$\alpha=20.0$	0.45	0.47	0.45
Number of ranked actives		49	44	31

^a Best results are highlighted in **bold italics** for each parameter.

protocols 2 (44) and **protocol 3** (31). However, a large number of decoys were also filtered out (1848 of 2900) for **protocol 3** and an increase in the statistics, particularly for the top-1% of database was observed. The use of the pharmacophore could increase the probability of identifying compounds that make necessary binding interactions with the hinge regions and have planar rings around the hinge region, so **protocol 3** was selected for screening.

2.2. Screening of the biogenic and analyticon discovery databases

The ZINC15 biogenic and Analyticon Discovery databases were combined, giving a total of over 68,000 compounds which were screened using the final protocol (**protocol 3**). The compounds were therefore pre-screened using the pharmacophore filter, with 17,267 compounds remaining possessing at least 3 of the 4 features (ADRR). This was followed by Glide-SP docking with constraints, which returned 11,593 unique compounds predicted to have binding affinity for CDK5 (**Scheme 1**). Compounds which ranked within the top ~ 1000 compounds were analysed and a total twenty-eight compounds were selected for *in vitro* validation. The compounds were assessed by the docking poses and ADME properties, considering potential blood-brain barrier permeability. A total of sixteen compounds (**1–16**) selected were flavonoids (**Table 5**) and the remaining twelve compounds (**Fig. 2**) with diverse scaffolds (**17–30**). Some flavonoids have already been shown to display inhibitory effects against CDK5 [**33–37**], therefore, a selection of flavonoid compounds were included in the *in vitro* CDK5/p35 binding assay validation for a structure activity relationship (SAR) analysis study. While fisetin (**1**) [**34**], apigenin (**6**) [**36**] and nepetin (**10**) [**33,37**] have previously been studied in isolated CDK5 enzyme binding assays (as discussed below), to our knowledge all other selected flavonoids in **Table 5** have not.

2.3. Inhibitor validation & analysis

The twenty-eight compounds were initially screened at a 50 μ M single concentration using a CDK5/p35 isolated enzyme binding assay to determine the percentage inhibition, and compounds with the highest percentage inhibition selected for IC₅₀ determination. The results of both of these are shown in **Table 6**, together with the original docking results and predicted log BB (logarithmic ratio between concentration of a compound in brain and blood) values. One sub-micromolar (fisetin) and five low micromolar flavonoid inhibitors (**2** gossypetin, **3** robinetin, **6** apigenin, **7** apigenin 7-sulfate, and **9** cirsiolil) with IC₅₀ < 10 μ M were identified, as well as three inhibitors with diverse chemical scaffolds. The predicted binding poses are shown in **Figs. 3 and 4**, respectively. Other notable inhibitors were flavonoid compounds **8** (5,7,2'-trihydroxyflavone, 76% inhibition at 50 μ M), but also **10–13** (nepetin, hispidulin, oroxylin A and jaceosidin, respectively) all of which showed moderate ~45–50% inhibition. Nepetin (**10**) has previously been reported to inhibit the kinase activity of a CDK5/p35 immuno-precipitate with IC₅₀ of 40 μ M [**33**], while an IC₅₀ of 3.172 μ M for CDK5/p25 has been reported [**37**]. A number of the selected candidates for experimental validation in **Table 6** revealed weak inhibition when tested in the binding assays, but false positives are an intrinsic feature of docking-based virtual screening [**38**]. This emphasizes limitations of docking (e.g. protein flexibility not adequately accounted for) and the importance of experimental validation of predictions.

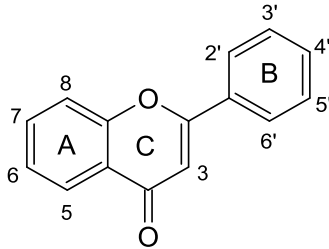
A SAR analysis was performed for the flavonoid compounds **1–16** (**Table 5**). Interest surrounding flavonoids for the inhibition of GBM has increased over recent years with a proposed synergistic effect with existing chemotherapeutic drugs such as TMZ [**39,40**]. Flavonoids such as rutin and those isolated from *Achyrocline satureioides* (marcela) extract in combination with TMZ have demonstrated beneficial effects such as increased apoptosis and reduced chemoresistance and proliferation against GBM cell lines and xenograft studies using BALB/c mice [**41,42**]. Jaceosidin (**13**) has been shown to inhibit the growth, survival, migration, and invasion of GBM T98G, with the expression of CDK5 reduced [**43**]. Recently, flavonoids such as EGCG [**44**] and baicalein [**45**] have also demonstrated promising anti-GBM potential.

Compounds **1–5** are a flavonol (3-hydroxyflavone backbone) subgroup of flavonoids. **1** (fisetin), **2** (gossypetin) and **3** (robinetin) had IC₅₀s < 10 μ M, whereas **4** (kaempferide) and **5** (morin) showed < 25% inhibition in the 50 μ M single concentration screening. The IC₅₀ of fisetin (0.90 μ M) is in line with previously reported data for CDK5/p25 (IC₅₀ = 0.57) [**34**]. Fisetin, gossypetin and robinetin all have a -OH group at the 3' position on ring B that is absent for kaempferide and morin, indicating the importance of this -OH group to potency. Fisetin and gossypetin are predicted to bind in a similar manner (**Fig. 3A and 3B**). Fisetin forms key hinge region hydrogen bonds with Glu81 backbone O and Cys83 backbone NH, with the -OH group at position 7 (ring A) forming a hydrogen bond with Asp86 sidechain carboxylate. The B ring is orientated towards the gate-keeper residue Phe80 where π - π stacking can occur, also enabling hydrogen bonding of ring B 4'-OH with Glu51 (α -C helix) and Lys33 (β -strand) sidechains as donor and acceptor, respectively. The binding is very similar to that of the solved CDK6-fisetin crystal structure (PDB ID: 1XO2), with fisetin having demonstrated similar potency against CDK6/Vcyclin (IC₅₀ of 0.85 μ M) [**34**]. Gossypetin has additional A-ring hydroxyl groups (5-OH and 8-OH) and was 6-fold less potent than fisetin (IC₅₀ of 5.73 μ M versus 0.90 μ M); the 5-OH allows hinge region hydrogen bonding with Cys83 backbone O, but resulted in a slight shift in position compared to fisetin affecting interactions (**Fig. 3B**). Compared to fisetin, robinetin only has an extra B-ring 5'-OH. This resulted in a ~11-fold drop in potency (IC₅₀ of 9.83 μ M versus 0.90 μ M). Glide-SP predicts an alternative binding orientation, but the predicted pose is arguably not convincing (**Fig. 3C**).

Compounds **6–16** belong to the flavone subgroup of flavonoids, differing from flavonols because they lack the 3-OH group. The most potent compounds of this subgroup (IC₅₀s ~ 1–3 μ M) were **6** (apigenin), **7** (apigenin 7-sulfate) & **9** (cirsiolil) with all having a 5-OH (ring A) and 4'-OH (ring B). Apigenin has previously been studied for CDK5 inhibition with similar potency reported (K_d = 3.9 μ M) [**36**]. The 7-OH to 7-OSO₃H ring A substitution for apigenin 7-sulfate had a ~3-fold effect on potency (IC₅₀ = 1.19 μ M vs 3.52 μ M). Predicted binding orientations of apigenin and apigenin 7-sulfate are shown in **Fig. 3D and 3E**, respectively. While apigenin binds with its B ring towards the gatekeeper Phe80 residue (and similar to gossypetin, **Fig. 3B**), apigenin 7-sulfate is in a flipped orientation with the sulfate positioned near Lys33, while still facilitating hinge region interactions. Solved crystal structures of CDK2 and CDK9 in complex with the synthetic flavonoid analogue flavopiridol (PDB IDs: 6GUB and 3BLR, respectively) show this orientation [**46,47**]. Compounds **9–13** all had a -OMe group at position 6 (ring A). While **9** (cirsiolil) was a potent inhibitor with an IC₅₀ of 3.55 μ M, **10–13** inhibited activity by only ~45–50%; the additional -OMe group substitution on ring A (position 7) rather than -OH appears to have led to the better



Scheme 1. Process of preparing the ZINC15 Biogenic and Analyticon Discovery databases for virtual screening indicating the number of unique compounds (excluding different tautomer and ionisation states) which were returned after each screening step.

Table 5Table of flavonoid compounds (1–16) selected for *in vitro* binding assay validation. Full chemical names are given in Table S2.


Compound	3	5	6	7	3'	4'	+OH
1 Fisetin	-OH	-	-	-OH	-OH	-OH	-
2 Gossypetin	-OH	-OH	-	-OH	-OH	-OH	8
3 Robinetin	-OH	-	-	-OH	-OH	-OH	5'
4 Kaempferide	-OH	-OH	-	-OH	-	-OCH ₃	-
5 Morin	-OH	-OH	-	-OH	-	-OH	2'
6 Apigenin	-	-OH	-	-OH	-	-OH	-
7 Apigenin 7-sulfate	-	-OH	-	-OSO ₃ H	-	-OH	-
8 5,7,2'-trihydroxyflavone	-	-OH	-	-OH	-	-	2'
9 Cirsiliol	-	-OH	-OCH ₃	-OCH ₃	-OH	-OH	-
10 Nepetin	-	-OH	-OCH ₃	-OH	-OH	-OH	-
11 Hispidulin	-	-OH	-OCH ₃	-OH	-	-OH	-
12 Oroxylin A	-	-OH	-OCH ₃	-OH	-	-	-
13 Jaceosidin	-	-OH	-OCH ₃	-OH	-OCH ₃	-OH	-
14 5,7,2',6'-tetrahydroxyflavone	-	-OH	-	-OH	-	-	2',6'
15 Chrysin-7-O-glucuronide	-	-OH	-	-O-Gluc	-	-	-
16 Kaempferitrin	-O-Rha	-OH	-	-O-Rha	-	-OH	-

potency. The predicted binding of cirsiliol is shown in Fig. 3F with hinge region hydrogen bonding conserved and the B-ring orientated towards gatekeeper residue, Phe80, while also exploiting hydroxyl substituent hydrogen bonds. Compounds 15 (chrysin-7-O-glucuronide) and 16 (kaempferitrin) have a sugar moiety at position 7 and kaempferitrin has an additional one at position 3. Both proved to be poor CDK5 inhibitors.

Compounds 17–28 had diverse scaffolds (Fig. 2). Of these compounds, 23, 25 and 27 (NP-018705, NP-009242, and Piperolactam A) were hits and their IC₅₀s determined as 42.6 μM, 46.5 μM and 26.7 μM, respectively. Their predicted binding is shown in Fig. 4. NP-018705 (1,7-dihydroxy-9-oxoxanthene-3-carboxylic acid, 23) has a xanthone-type scaffold, polycyclic aromatic type compounds containing a xanthone moiety conjugated to a ketone group at carbon 9. Nauman and colleagues (2021) published a study of nine xanthenes which inhibit CDK2/CyclinE1 [48]. The compounds were evaluated for their effect on breast and prostate cancer cell lines, revealing concentration-dependent inhibition for all compounds. Here, the inhibitor is predicted to form hydrogen bonds with the hinge region (Cys83 backbone O and NH) and Asn144 belonging to the activation loop (Fig. 4A). There are ion-ion interactions with Lys33 and π - π interactions with the gatekeeper residue, Phe80. Compound 25, 1-(7-hydroxy-9H-pyrido[3,4-b]indol-1-yl)ethan-1-one (NP-009242) is a β -carboline, an indole alkaloid having a tricyclic pyrido[3,4-b]indole ring in its structure, considered a promising lead scaffold for design of anticancer agents. β -carboline have varied anticancer targets such as DNA, enzymes (such as topoisomerases and kinases) and proteins (such as tubulin and ABCG2/BRCP1) [49]. They have been reported as specific inhibitors of CDKs [50] and in recent years have shown promise against glioma and GBM [51–53]. The predicted binding of 25 (Fig. 4B) reveals the compound binds at the ATP-binding site with three hinge region hydrogen bonds, and ample space near gatekeeper residue Phe80 for suitable substituents to exploit favourable interactions to improve potency and selectivity. Compound 27, Piperolactam A, is a tetracyclic alkaloid and an aristolactam derivative found in many plants. Aristolactam variants have been shown to induce cancer cell death and inhibit cancer cell proliferation [54,55]. Aristolactam BIII is a highly potent low nanomolar inhibitor of dual-specificity tyrosine phosphorylation-regulated kinase 1A

(DYRK1A) [56]; DYRK1A inhibition has attracted recent interest as a promising translational opportunity to sensitize GBM cells to FDA-approved cancer therapeutics [57]. The docking of Piperolactam A at the CDK5 ATP-binding site (Fig. 4C) revealed two hinge region hydrogen bonds with Glu81 and Cys83 backbones, and π - π interactions with the gatekeeper residue. Again, investigating analogues of this compound to improve its potency (IC₅₀ = 26.73 μM) and selectivity appears justified.

The potential for CNS-activity of the most potent identified flavonoids, fisetin (1) gossypetin (2), robinetin (3), apigenin (6), apigenin 7-sulfate (7), cirsiliol (9) with IC₅₀ < 10 μM was considered using log BB predictions, where all values except for fisetin, apigenin and cirsiliol were < −2 (Table 6). These three compounds had values in the range −1 to −2, with a value greater than −1 desirable for potential CNS-activity [58–60]. However, the accuracy of the predicted log BB values for the flavonoid polyphenol-type chemotype is not clear, especially given that fisetin is known to show blood-brain barrier permeability [61]. A pharmacokinetic limitation of flavonoids reaching the brain is low systemic bioavailability. Several strategies are being investigated to improve flavonoid blood-brain barrier permeability, particularly nanocarrier-based systems such as lipid nanoparticles, liposomes, polymeric nanoparticles and nanoemulsions, which can improve flavonoid stability, bioavailability and targeted delivery [62,63]. In contrast, for the three hit compounds with diverse scaffolds (23, 25 and 27), both 25 and 27 revealed log BB values of ~ −0.7, consistent with potential for CNS activity and providing encouragement for further optimization studies.

2.4. MD simulation: CDK5 – fisetin complex

To gain a better understanding of the protein-ligand interactions that led to the observed sub-micromolar potency of fisetin (1), a 1 μs MD simulation was performed using the top-ranked Glide-SP docked pose of the compound with CDK5 (PDB code: 3O0G). The RMSD backbone and sidechain plots included in Figure S1 demonstrate that the system quickly reached equilibrium and was stable. Fisetin remained tightly bound at the ATP-binding site throughout the simulation, with the

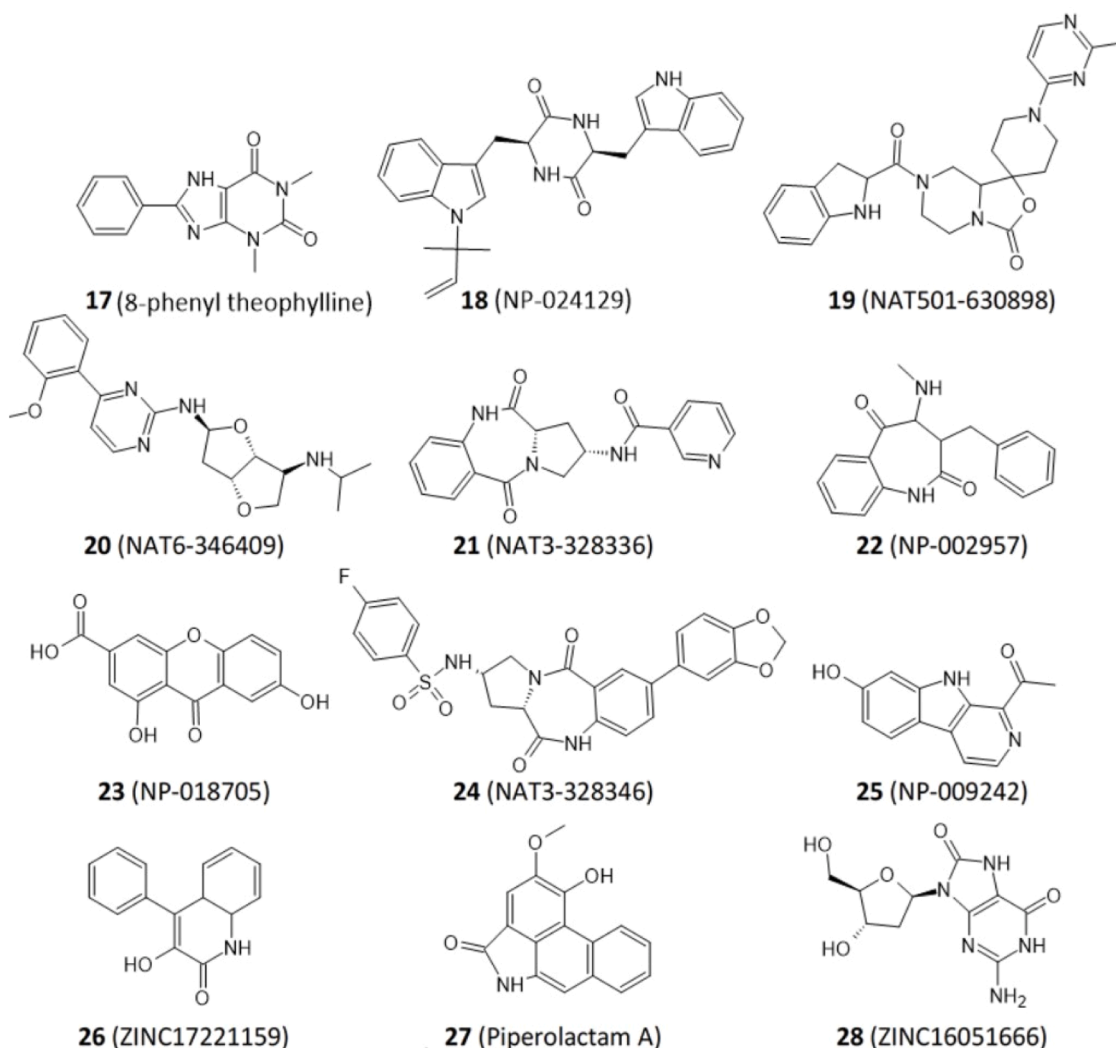


Fig. 2. Structures of compounds 17–28 selected for *in vitro* binding assays, together with their ZINC IDs. Full chemical names are given in Table S2.

prevalence of protein-ligand interactions for the last 900 ns included in Fig. 5. The hydrogen bonding with hinge region Glu81 O and Cys83 NH backbone atoms is highly conserved (~98% duration), as are the hydrogen bond interactions with Asp86 sidechain carboxylate involving the fisetin A-ring hydroxyl group (90% duration). There were strong hydrogen bonding interactions involving the B-ring 4'-OH with Lys33 sidechain (78% duration), while there are also direct interactions of this group with Glu51 (α -C helix) sidechain carboxylate, these interactions are predicted to be mainly water-bridged. The B-ring 3'-OH is also involved in hydrogen bonding (38% duration) with Asn144 sidechain CO group (wild-type has Asp144). There were ligand hydrophobic interactions with Ile10, Val18, Phe80 (gatekeeper π - π stacking, T-shaped) and Leu133 residues. As part of the Supporting Information, a MD simulation movie of fisetin binding at the ATP-binding site of CDK5 has been included.

2.5. Kinase selectivity screening

Due to the highly conserved nature of the ATP-binding site of kinases, selectivity is a limiting factor for kinase inhibitors. However, it has been shown that selectivity can be achieved by targeting heterogeneity in conformationally flexible regions within the binding site [64]. The homologous kinases used for selectivity screening were CDK5, CDK2, CDK9, GSK-3 β , and ERK2, all of which are found in the brain and play roles in GBM tumorigenesis [65–68]. These kinases have a close

similarity to CDK5 (Figure S2) and also play roles in the progression of neurodegenerative disorders, as well as GBM [69–71].

Fisetin is a potent CDK inhibitor displaying nanomolar activity against CDK1, CDK5, CDK6, as well as GSK-3 β [34], and low μ M inhibition against CDK2 ($IC_{50} \sim 2$ – 3μ M) [72]. It is also a receptor tyrosine kinase c-Kit ($IC_{50} = 1.34 \mu$ M) inhibitor [72]. Seven of the other most potent compounds identified here, gossypetin (2), robinetin (3), apigenin 7-sulfate (7), cirsiol (9), NP-018705 (23), NP-009242 (25) and Piperolactam A (27) were screened at a single concentration of 50μ M against the five kinases to identify if selectivity towards CDK5 is observed. Table 7 shows the percentage inhibition of each compound against the screened kinases. For the flavonoids, the most selective compound was cirsiol in terms of CDK5 inhibition (95.8%) in comparison to the other kinases (CDK2: 81.6%, CDK9: 59.3%, GSK-3 β : 62.2%, ERK2: 22.6%). The effects of cirsiol have not previously been studied for GBM, however, one study in 2008 reported promising effects against breast cancer cell lines, demonstrating its cancer therapeutic potential [73]. Gossypetin was also relatively CDK2 and CDK5 selective, with CDK2 inhibition (96.5%) over CDK5 (82.5%). Robinetin showed high but broad inhibition for CDK5 (81.0%), CDK2 (91.0%), GSK-3 β (98%) and ERK2 (95.2%), but lower for CDK9 (56.8%). Apigenin 7-sulfate and the three novel compounds NP-018705, NP-009242 and Piperolactam A showed inhibition of CDK5, CDK2, CDK9 and GSK-3 β and no or limited inhibition of ERK2. The increased inhibition for NP-018705 in CDK2 compared to CDK5 (78.8% vs 44.0% inhibition)

Table 6

Glide-SP docking scores and log BB values together with the experimental *in vitro* CDK5/p35 binding assay results for compounds 1–28 selected from *in silico* screening.

Compound ^a	Computational results ^b			Experimental
	Docking score	Docking rank	Log BB	
1	−8.371	528	−1.91	0.90 ± 0.21
2	−8.122	723	−2.77	5.73 ± 1.95
3	−8.404	500	−2.40	9.83 ± 2.02
4	−8.167	681	−1.36	(24.3%)
5	−7.987	855	−2.25	(12.2%)
6	−8.947	186	−1.45	1.19 ± 0.29
7	−9.008	165	−2.61	3.52 ± 0.10
8	−8.089	762	−1.29	(75.7%)
9	−8.842	236	−1.51	4.73 ± 1.66
10	−8.707	298	−1.84	(49.6%)
11	−8.971	177	−1.34	(48.5%)
12	−8.540	393	−0.77	(46.6%)
13	−8.986	171	−1.28	(45.2%)
14	−8.549	381	−1.74	(33%)
15	−8.911	203	−2.87	(8.7%)
16	−9.170	106	−4.19	(NI) ^c
17	−9.009	164	−0.51	(NI) ^c
18	−8.925	196	−1.28	(NI) ^c
19	−9.387	66	−0.74	(NI) ^c
20	−9.144	111	0.11	(NI) ^c
21	−9.793	23	−1.03	(NI) ^c
22	−9.133	113	−0.35	(NI) ^c
23	−9.366	68	−1.76	42.61 ± 0.67
24	−8.800	256	−1.31	(NI) ^c
25	−9.038	148	−0.67	46.52 ± 18.51
26	−7.841	1044	−0.61	(12%)
27	−8.626	336	−0.69	26.73 ± 4.82
28	−8.754	279	−2.55	(NI) ^c

^a Chemical structures shown in Table 5 and Fig. 2. ^b Docking scores and ranks from Glide-SP; Log BB values from QikProp. ^c No inhibition.

links back to the study discussed earlier for the xanthone scaffold which reported hydroxyl groups as beneficial functional groups for CDK2 inhibition [48]. This preliminary selectivity screen of homologous kinases would justify future *in vitro* screening of a wider enzyme panel to establish any off-target effects.

2.6. *In vitro* glioblastoma assays

2.6.1. Initial single concentration screen

An initial single concentration screen (100 μ M) was carried out on the selected hit compounds; fisetin (1), gossypetin (2), robinetin (3), apigenin (6), apigenin 7-sulfate (7) and cirsiolol (9), all of which were flavonoid compounds identified with low micromolar potency (< 10 μ M) in the *in vitro* CDK5 isolated binding assays (Table 6). Cells were treated after 24 h of incubation and then screened following 24-, 48-, and 72- hours, with the results shown in Fig. 6. With the exception of the T98G experiments, all compounds showed a time-dependent effect on cell viability. Fisetin was the most effective compound at reducing cell viability across all three cell-lines and was taken forward for IC₅₀ profiling. Both apigenin and apigenin 7-sulfate reduced cell viability of all cell lines to <50% of control following 72 h, therefore, they were also selected for further assessment. When the results of cirsiolol and gossypetin were compared, they showed similar efficacy against the U87-MG and T98G cell lines. However, gossypetin was selected over cirsiolol considering U251-MG effects, reducing cell viability to 27.4% versus 48.5% at 72 h, respectively. In the interest of only screening compounds likely to show efficacy at lower concentrations, robinetin was also not taken forward.

2.6.2. IC₅₀ determinations

The initial single concentration screen of the six compounds against the three GBM cell lines revealed the most potent effects were for fisetin,

gossypetin, apigenin and apigenin 7-sulfate. Subsequently, the IC₅₀s of these four compounds were determined (Table 8 and Fig. 7).

Further evaluation of compound 1 (fisetin) against each of the cell lines to determine IC₅₀ values revealed both concentration and time dependent effects on viability (Fig. 7A). As the initial screen revealed only minor reductions in cell viability following 24 h of treatment, IC₅₀s were determined following 48 and 72 h of treatment. Treatments were 1, 3, 10, 30, 100 and 300 μ M. Post-hoc analysis showed that at 30 μ M, significant effects were observed in U87-MG after 48 and 72 h ($p < 0.05$), U251-MG after 48 and 72 h ($p < 0.001$), and T98G after 48 h ($p < 0.001$) and 72 h ($p < 0.005$). Additionally, treatment at 10 μ M significantly reduced U251-MG viability after 48 h ($p < 0.001$). Following 72 h of treatment, the IC₅₀ values were determined as 40.6, 32.1 and 22.2 μ M in U87-MG, T98G and U251-MG cell lines (Table 8), respectively. In comparison, the well-known CDK inhibitor roscovitine has IC₅₀'s ~15–19 μ M against the same cell lines [10], with fisetin as a naturally occurring polyphenol showing close to similar efficacy. As mentioned in the introduction, TMZ resistance is common among GBM patients due to a MGMT positive status. The T98G cell line is MGMT positive, so identification of compounds such as fisetin that show promise against the T98G cell line could be beneficial with respect to overcoming therapeutic resistance. Consistent with this, fisetin has very recently shown potential to reverse both inherited and acquired TMZ resistance in other studies by downregulating ZEB1, a transcription factor responsible for tumor cell migration and drug evasiveness [74]. Fisetin may also enhance the effect of anticancer drugs and help reduce therapy-induced senescent cells [75].

Treatment of the three cell lines with compound 2 (gossypetin) to determine IC₅₀ values revealed both concentration and time dependent effects on cell viability (Fig. 7B). For example, at 30 μ M, viability was significantly reduced in U251-MG after 48 and 72 h ($p < 0.001$). The flavonol was predicted to bind in a similar orientation to fisetin (Fig. 3A and 3B), but was less potent against CDK5/p35 (Table 6). Gossypetin displayed similarly good efficacy against the U251-MG cell line (IC₅₀ = 28.8 μ M at 72 h) and moderate efficacy against U87-MG (IC₅₀ = 105.9 μ M at 72 h) (Table 8). A reproducible decrease in efficacy of gossypetin was observed following 72 h of treatment in the T98G cell line across the three independent experiments. While the mechanism of this resistance was beyond the scope of this study, the T98G cell line is known to demonstrate greater treatment resistance due to its MGMT status. MGMT expression is regulated by nuclear factor- κ B (NF- κ B) signalling, and while flavonoids often regulate this pathway and thus decrease MGMT expression, gossypetin does not inhibit activation of NF- κ B [76]. This result highlights the importance of fisetin which remained efficacious at 72 h in the same cell line.

Compounds 6 (apigenin) and 7 (apigenin 7-sulfate) both belong to the flavone subgroup of flavonoids. As with fisetin, apigenin showed limited efficacy at 24 h across the three cell lines but reduced cell viability of both T98G and U251-MG in a concentration dependent manner following 48 and 72 h of treatment (Fig. 7C). At the highest treatment concentration, apigenin reduced viability following 48 and 72 h of treatment in T98G ($p < 0.001$ and 0.05 respectively) and U251-MG ($p < 0.01$ and 0.001 respectively). Following 72 h of treatment, the IC₅₀ were determined as 141.2 and 121.8 μ M (Table 8), respectively. It was less effective against U87-MG (IC₅₀ = 506.6 μ M at 72 h). Compound 7 (apigenin 7-sulfate) showed ~3-fold less potency for CDK5/p35 inhibition compared to apigenin (Table 6) but both were low micromolar inhibitors. It was found to reduce cell viability at 24, 48 and 72 h in a concentration and time dependent manner across all three cell lines (Fig. 7D). Analysis showed that at 100 μ M, significant effects were observed in U87-MG after 72 h ($p < 0.05$), T98G after 48 and 72 h ($p < 0.001$), and U251-MG after 48 ($p < 0.001$) and 72 h ($p < 0.05$). The IC₅₀ were determined as 333.2 and 206.5 μ M at 72 h in U87-MG T98G and U251-MG cell lines (Table 8), respectively. It was most effective against the MGMT positive T98G (IC₅₀ = 57.32 μ M at 72 h). From the kinase selectivity screening (Table 7), its action is that of a MKI

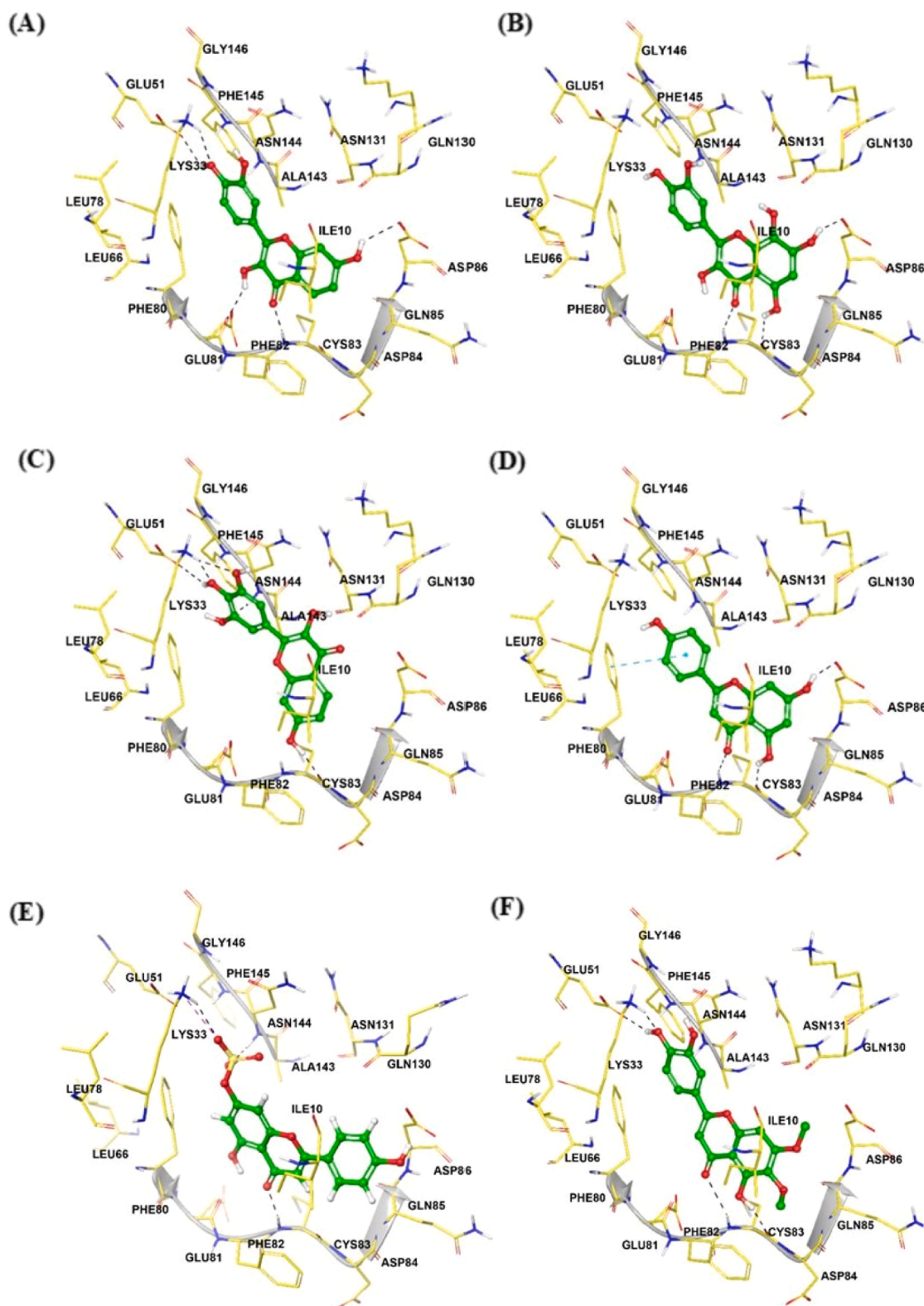


Fig. 3. The docked orientations and predicted interactions of the identified flavonoid inhibitor compounds with $IC_{50} < 10 \mu M$ at the ATP-binding site of CDK5. (A) 1, fisetin ($IC_{50} = 0.90 \mu M$), (B) 2, gossypetin ($IC_{50} = 5.73 \mu M$), (C) 3, robinetin ($IC_{50} = 9.83 \mu M$), (D) 6, apigenin ($IC_{50} = 1.19 \mu M$), (E) 7, apigenin 7-sulfate ($IC_{50} = 3.52 \mu M$), and (F) 9, cirsiolil ($IC_{50} = 3.55 \mu M$). Fisetin was most potent, in the sub-micromolar range. Hydrogen bond interactions between the protein and ligand are shown as black dashed lines, π - π interactions shown as cyan dashed lines.

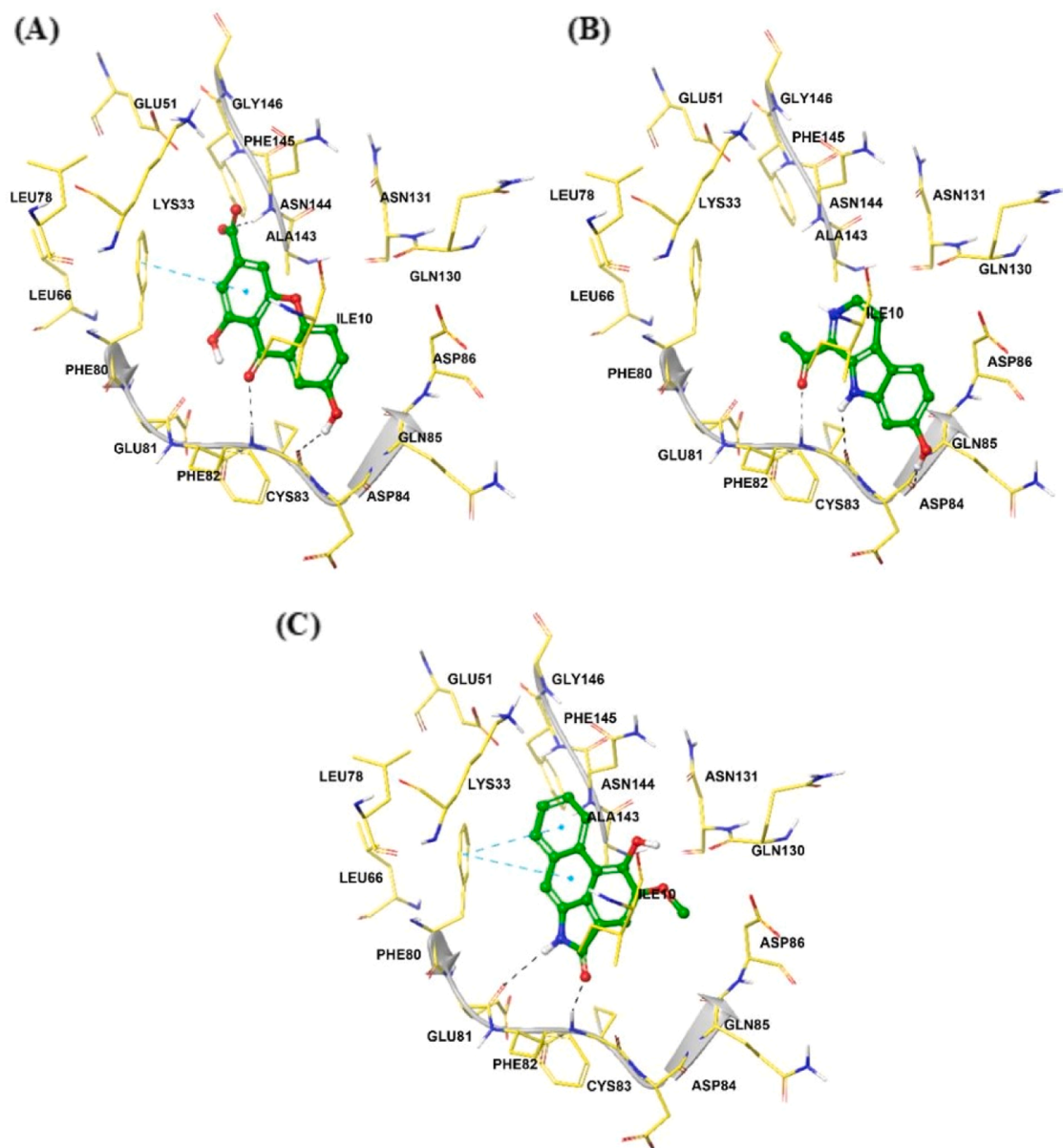


Fig. 4. The docked orientation and predicted interactions of the identified micromolar inhibitor compounds with diverse scaffolds at the CDK5 ATP-binding site. (A) **23**, NP-018705 ($IC_{50} = 42.61 \mu M$), (B) **25**, NP-009242 ($IC_{50} = 46.52 \mu M$), and (C) **27**, Piperolactam A ($IC_{50} = 26.73 \mu M$). Hydrogen bond interactions represented as black dashed lines, π - π shown as cyan dashed lines.

compound, appearing most potent against GSK-3 β and CDK9 for the kinases studied.

3. Conclusions

Discovery of drug-like CDK5 inhibitors for the potential treatment of GBM has significant promise given CDK5's role in signalling pathways that promote tumour growth and invasion, its contribution to therapy resistance, and influence on GSCs and the tumour microenvironment (TME). In this study, we have used an *in silico* motivated approach for identification of CDK5 inhibitors, screening the ZINC15 biogenic set and an Analyticon Discovery natural product database. Twenty-eight candidate inhibitors were selected and validated using *in vitro* CDK5/p35 isolated enzyme binding assays, including sixteen flavonoids and twelve structurally diverse compounds. This resulted in the successful identification of six low micromolar flavonoid inhibitors, with fisetin (**1**), gossypetin (**2**), robinetin (**3**), apigenin (**6**), apigenin 7-sulfate (**7**),

and cirsiol (**9**) showing $IC_{50}s < 10 \mu M$, and fisetin the most potent ($IC_{50} = 0.90 \mu M$). Flavonoids have a range of promising biological activities from anti-inflammatory, antibacterial, antiviral, antidiabetic, antioxidant, neuroprotective, as well as anticancer, making them promising drug leads for development [77]. SAR analysis of the sixteen flavonoid structural features identified B ring 3'-OH substitution as important for flavonol sub-group potency, with an additional B ring 5'-OH not favourable. The most potent flavones all had 5-OH (ring A) and 4'-OH (ring B) substitutions, and while $-OCH_3$ substitution at ring A position 6 alone appeared detrimental to potency, $-OCH_3$ substitution at both positions 6 and 7 was effective for potency and kinase selectivity, as observed for cirsiol ($IC_{50} = 3.55 \mu M$). Additionally, three hit compounds with diverse chemical scaffolds were identified, consisting of xanthone, aristolactam and β -carboline cores that can be explored for kinase inhibition and selectivity potential through further optimization studies. Compounds based on these scaffolds have previously demonstrated anti-cancer potential, as highlighted earlier. The most potent

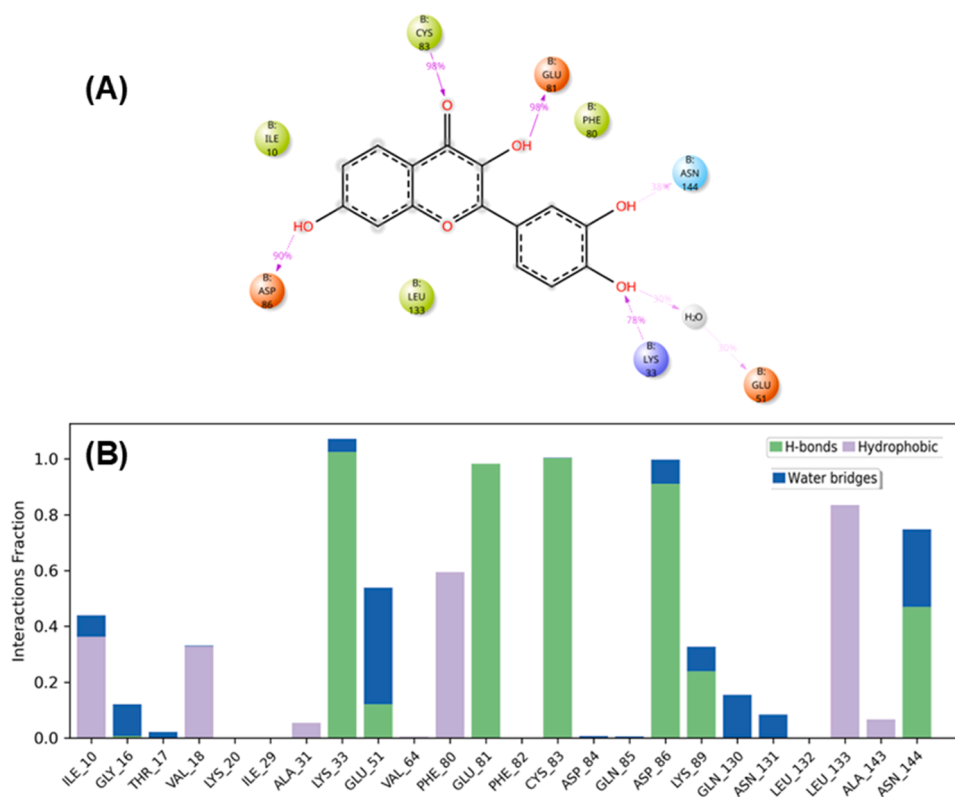


Fig. 5. Molecular dynamics simulation of the CDK5 – fisetin (1) complex. (A) Interactions with key ATP-binding site residues and fisetin during the simulation, with % prevalence of hydrogen bonds shown and (B) Interaction fraction of fisetin with a broader range of ATP-binding site residues.

Table 7

Kinase selectivity profiling for identified inhibitors shown as the % inhibition at 50 μ M against five homologous kinases. The % inhibition of each compound against CDK5/p35 was used as a benchmark.

Compound	% inhibition at 50 μ M				
	CDK5	CDK2	CDK9	GSK-3 β	ERK2
2 Gossypetin	82.5	96.5	19.9	52.8	20.1
3 Robinetin	81.8	91.0	56.8	98.0	95.2
7 Apigenin 7-sulfate	68.1	69.4	84.9	96.6	15.2
9 Cirsiliol	95.8	81.6	59.3	62.2	22.6
23 NP-018705	44.0	78.8	69.4	59.8	NI ^a
25 NP-009242	42.5	68.8	49.0	59.2	NI ^a
27 Piperolactam A	41.2	76.3	46.2	35.8	NI ^a

^a No inhibition.

compounds, six flavonoids, were tested for their anti-GBM potential by *in vitro* cellular studies against three cell lines. Cell viability single concentration screening led to identification of fisetin, gossypetin, apigenin and apigenin 7-sulfate as the most promising and following IC₅₀ determination, the flavonol fisetin emerged as having the most potent effects (IC₅₀ ~ 20–40 μ M at 72 h). In this study, we did not look at, for example, phospho-CREB1 levels (CREB1, a transcription factor and CDK5 substrate) which would be interesting in future work given that CDK5-CREB1 pathway suppresses both self-renewal of GSCs and glioma growth [16]. The GBM experimental findings presented in this study are *in vitro*, with the efficacy and translational relevance not validated *in vivo*. However, fisetin potential as an adjuvant therapy for GBM is gathering momentum, particularly in the last couple of years [74,75,78]. Fisetin is able to cross the blood brain barrier [61], but its lower bioavailability needs to be addressed [78,79]. Promisingly, in studies of different cancers, fisetin has shown synergistic effects in conjunction with other anticancer agents, including for *in vivo* experiments [80].

4. Experimental

4.1. Computational details

4.1.1. Ligand preparation

Native crystallographic ligands: The cognate ligands of the five CDK5 protein-ligand complexes from the PDB database (Table 1) were prepared using LigPrep 5.6[81] and the OPLS3e forcefield[82] at target pH 7.0 $\pm$ 2.0, with the different tautomeric and ionisation states generated.

Creation of the active/decoy ligand set: Known potent active ligands for CDK5 inhibition were extracted from the ChEMBL database (<https://www.ebi.ac.uk/chembl/>) [83]. A total of 620 ligands which had a potency of < 500 nM for CDK5 were clustered based on diversity (DISE (Direct Sphere Exclusion) method) using Canvas [81], splitting the ligands into fifty structurally diverse groups with a representative for each subset selected as an active. To generate the decoy set, the SMILES of the fifty representative actives were then inputted into the DUD-E decoy generator (<https://dude.docking.org/>), which generated 58 files with each containing fifty decoy ligands. A total of 2900 decoys were produced and prepared for docking along with the actives using LigPrep 5.6.

Screening database: The screening databases used were the ZINC15 Biogenic database (<https://zinc15.docking.org/>)[26] and the Analyticon Discovery (AD) database (www.ac-discovery.com). The compounds were initially downloaded from their respective websites and were then prepared using LigPrep 5.6. The OPLS3e forcefield was used and possible ionization and tautomeric states generated using Epik at a target pH 7.0 $\pm$ 2.0. Log BB values of the selected candidates for experimental validation were calculated using QikProp 6.8 [81].

4.1.2. Protein target preparation and selection

Five solved crystal structures of human CDK5 were downloaded from the PDB database with inhibitors bound at the ATP-binding site (PDB

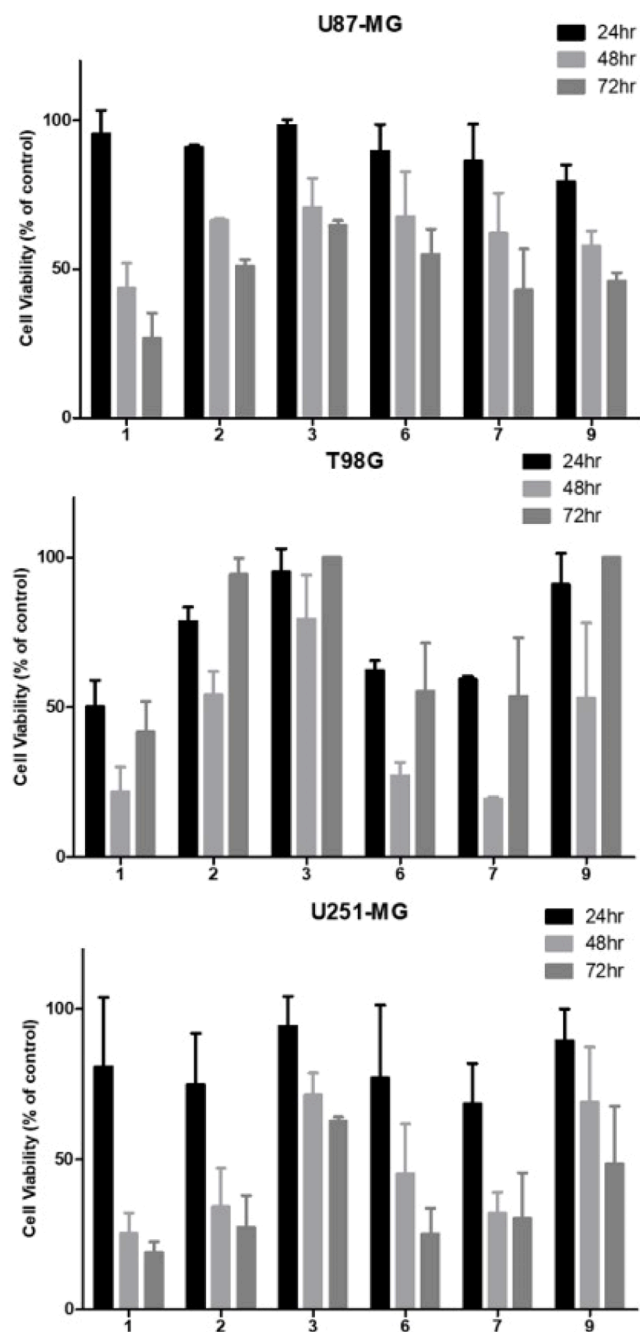


Fig. 6. Cell viability of the three glioblastoma cell lines U87-MG, T98G, and U251-MG at 24-, 48- and 72-hours following treatments at 100 μ M concentrations with the CDK5/p35 hit flavonoid compounds: fisetin (1), gossypetin (2), robinetin (3), apigenin (6), apigenin 7-sulfate (7) and cirsiolol (9). Data obtained from mean $\pm$ SEM of three consecutive independent experiments.

codes: 3OOG, 1UNG, 1UNH, 1UNL, 4AU8) [84]. The Schrödinger 'Protein Preparation Wizard' [81] was used to prepare the proteins with water molecules within 5.0 Å of ligands initially retained, hydrogens added and bond orders assigned. Protein residue protonation states were optimised using PROPKA [85,86] at the default pH 7.0 and the protein-ligand complexes minimised using the OPLS3e forcefield to converge heavy atoms with a maximum RMSD of 0.3 Å. Of the CDK5 structures used for analysis, 3OOG, 1UNG, 1UNH and 1UNL were CDK5/p25 complexes which is the kinase in complex with one of its shortened co-activator proteins which is most common in disease [87].

Table 8

Cell viability IC₅₀ values for the glioblastoma cell lines, U87-MG, T98G and U251-MG, following 24-, 48-, and 72-hour treatments with compounds 1, 2, 6 and 7.

Cell line	IC ₅₀ (μ M)		
	24hr	48hr	72hr
1 (fisetin)			
U87-MG	-	69	40.6
T98G	-	25	32.1
U251-MG	-	28.4	22.2
2 (gossypetin)			
U87-MG	384.3	159.8	105.9
T98G	2732.0	59.0	824,300.0
U251-MG	374.8	26.4	28.8
6 (apigenin)			
U87-MG	-	-	506.6
T98G	-	67.2	141.2
U251-MG	-	537.5	121.8
7 (apigenin 7-sulfate)			
U87-MG	551.5	645.1	333.2
T98G	390.6	80.93	57.32
U251-MG	353.5	264.4	206.5

The remaining CDK5 structure used, 4AU8, was noted to be without an activator protein bound in complex [30]. All the structures had a native ligand bound that was a potent CDK5 inhibitor (Table 1). The ATP-binding site volume of each of the five PDB's was calculated using SiteMap [81].

4.1.3. Docking calculations

Schrödinger's Glide 9.1 [81] was used for docking. The receptor grid was first generated and mapped using the centroid of the native ligands and had dimensions $\sim 24 \times 24 \times 24$ Å for the PDB proteins used. Docking calculations were performed in either -SP or -XP mode as indicated in the results, with no waters present. For all Glide docking calculations, the OPLS3e forcefield was used and the ligand van der Waals (vdW) radii scaling factor left to default of 0.80 and partial charge cut off at 0.15. The ligand sampling was set to flexible with both sample nitrogen inversions and sample ring conformations selected, along with Epik state penalties. Post-docking minimisation calculations were carried out on the output poses and strain correction terms applied. One pose per ligand was saved and the ligands were ranked based on docking score. Comparison of the docking approaches (benchmarking study) were based on the statistical analysis. Statistical performance metrics EF, EF', DEF, DEF' and BEDROC(alpha=20)[88] were used to compare docking protocol performances for recognition of actives.

To assess potential to improve results, constraints were also implemented during docking. Hinge region hydrogen bonding is crucial for potent ATP-binding site inhibitors [89]. During grid generation, hydrogen bond constraints were selected for hinge region Glu81 backbone O and Cys83 backbone NH and O. For Glide-SP, docking was performed with and without (for comparison) these hydrogen bond constraints as highlighted in Fig. 1A.

4.1.4. Pre-screening pharmacophore model

A 3D-pharmacophore model was generated using Schrödinger's program Phase 6.7 [39,81]. The five PDB protein-ligand complexes analysed in the benchmark study were first aligned using the hinge region backbone (residues 81–83), the protein was then deleted to leave the ligands superimposed. A 4-point pharmacophore was generated based on common structural features from the five native ligands.

4.1.5. Molecular dynamics details

MD simulations were performed using Desmond GPU [90]. The initial set-up of the CDK5/p25-fisetin (1) complex for MD simulation used the top-ranked Glide-SP docking pose. The system was first soaked with a

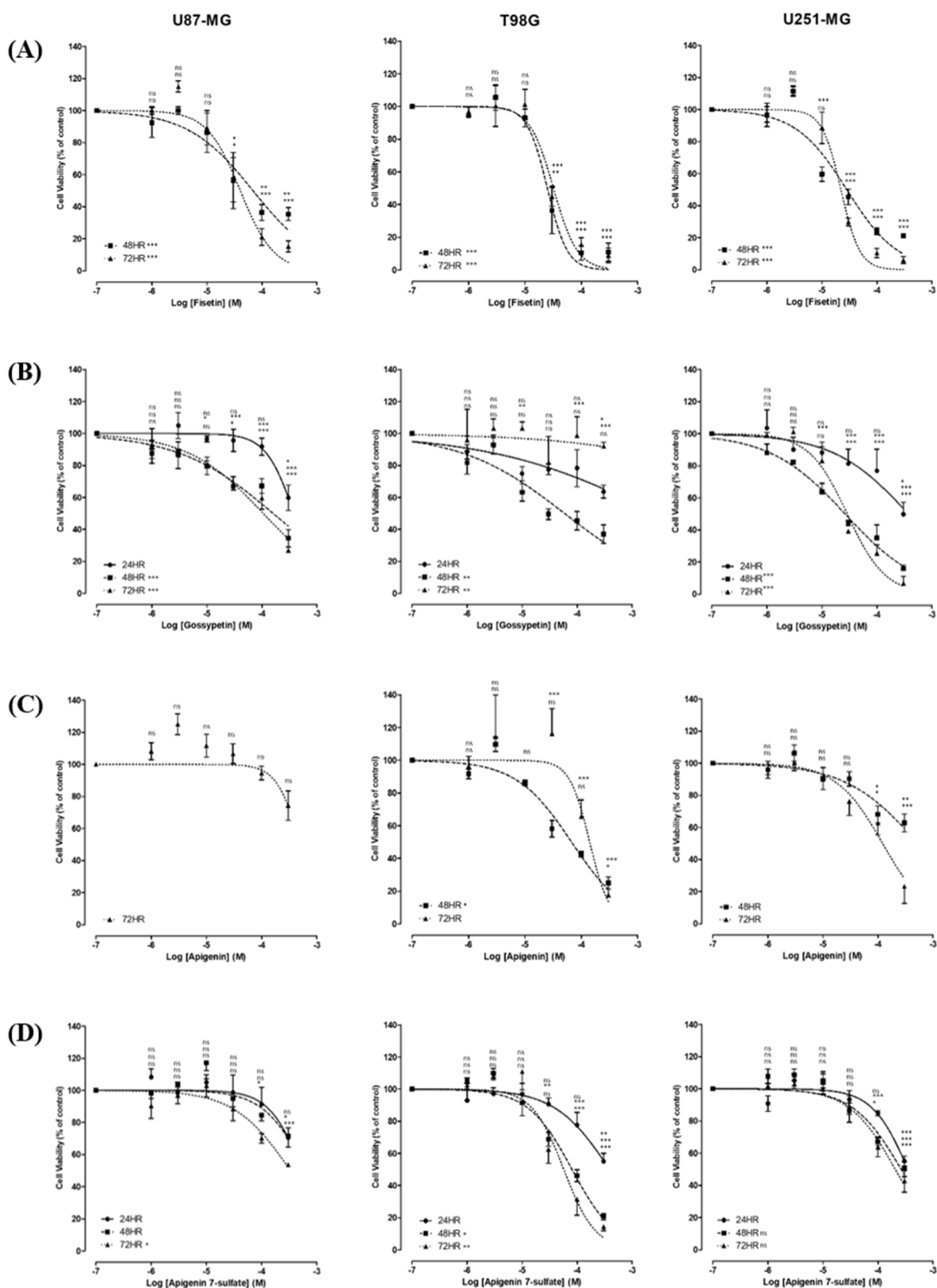


Fig. 7. Cell viability of the three glioblastoma cell lines U87-MG, T98G, and U251-MG following treatment with (A) compound 1 (fisetin) at 48 and 72-hours; (B) compound 2 (gossypetin) at 24, 48 and 72-hours; (C) compound 6 (apigenin) at 48 and 72-hours; and (D) compound 7 (apigenin 7-sulfate) at 24, 48 and 72-hours. Data obtained from mean $\pm$ SEM of three consecutive independent experiments. 24-hour results were omitted for compound 1 (fisetin) as no effect was seen. Significant effect observed between different time points is noted beside the legend (24-hours compared to 48 and 72-hours), while significant effect observed between the treatment concentration and the control is noted above the individual concentration points.

pre-equilibrated TIP3P water model (14,539 water molecules) orthorhombic box with volume 524,673 Å³ and a 10 Å buffer region between protein and box sides. The systems were neutralized with five Cl⁻ ions, with the final system consisting of 50,376 atoms. Water molecules internal geometries and heavy atom bond lengths with hydrogens were constrained with the SHAKE algorithm. The OPLS4 forcefield was used, together with a 9.0 Å cut-off for non-bond interactions, and periodic boundary conditions (PBC). Electrostatic interactions were treated with the Particle Mesh Ewald (PME) method. The simulation system was initially relaxed using the default standard Desmond relaxation/equilibration protocol, and this protocol was followed by a 1 μs production run in the NPT ensemble (T = 300 K, barostat relaxation time = 2.0 ps, thermostat relaxation time = 1.0 ps; P = 1 atm), using the Martyna-Tobias-Klein barostat and Nose-Hoover thermostat [91,92]. The multiple timestep RESPA integrator with timesteps 2, 2, and 6 fs for bonded, 'near' nonbonded and 'far' nonbonded interactions was used, respectively. Trajectory atomic coordinates and energy data were saved every 250 ps and 1.2 ps, respectively.

4.2. Experimental details

4.2.1. *In vitro* isolated enzyme binding assays

The *in vitro* validation of the candidate compounds involved isolated CDK5/p35 binding enzyme assays carried out by the 'International Centre for Kinase Profiling' at the University of Dundee (<https://www.kinase-science.mrc.ac.uk/>). All experiments were performed in duplicate. CDK5[1–292] used was full length, substrate histone H1 and the ATP concentration 20 μM. The compounds tested were purchased from Mcule, Biosynth (Carbosynth) or Analyticon Discovery (Table S2) and used as received. Initially, single concentration assay screening of the purchased compounds at 50 μM against full length CDK5/p35 was performed to determine the % inhibitions. Selected candidates which showed the best inhibition were selected for IC₅₀ determination with a top concentration of 200 μM, and final values reported as IC₅₀ ± SD (standard deviation). Selected most potent candidates were also screened against a panel of kinases (CDK2, CDK5, CDK9, ERK2, and GSK-3β) at a single concentration (50 μM) to gain an insight into the selectivity profile of these compounds.

4.2.2. *In vitro* glioblastoma assays

The effects of compounds on cell viability in three glioblastoma cell lines (U87-MG, U251-MG and T98G) were studied. U87-MG and T98-G cell lines were from the American Type Culture Collection, with U251-MG provided by University of Wolverhampton. Cell lines were maintained in Eagles Minimum Essential Media (EMEM), supplemented with 1% Non-Essential Amino Acids (NEAA), L-glutamine (2 mM), sodium pyruvate (1 mM) and 10% Foetal Bovine Serum (FBS). All the latter were purchased from Gibco. Cells were cultured at 37 °C in a humidified atmosphere maintained at 5% CO₂, were seeded using a density of 1 × 10³ cells per well in 96-well plates and cultured for 24 h prior to treatment with compounds (1–300 μM). Concentration of DMSO vehicle used was < 0.5% and was not cytotoxic to cells. Cell viability measurements were after 24-, 48- and 72- hours of treatment, using PrestoBlue® at an excitation/emission of 535/612 nm. All experiments were performed in triplicate, with mean ± SEM determined from three consecutive independent experiments. For statistical analysis, SPSS was used with a two-way ANOVA and a Bonferroni post-hoc test to identify significant differences between experimental groups. *P*-values ≤ 0.05 were considered statistically significant. Significance was indicated in figures as: ns (not significant), *p* > 0.05, * *p* ≤ 0.05, ** *p* ≤ 0.01, and *** *p* ≤ 0.001.

CRediT authorship contribution statement

Zahra R. Khan: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Philip J. Welsby:** Writing – review & editing, Visualization, Supervision, Methodology, Formal analysis.

Izabela Stasik: Supervision, Methodology. **Joseph M. Hayes:** Writing – review & editing, Visualization, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Z.R.K. and J.M.H. gratefully acknowledge funding and support from the Sydney Driscoll Neuroscience Foundation (SDNF).

Supplementary materials

Supplementary tables and figures from calculations; ZINC codes, vendors, purities, chemical names and formulae, molecular weights and strings formulae (SMILES) of compounds 1–28. The molecular dynamics trajectory for fisetin binding at the CDK5 ATP-binding site is also provided as a supplementary video (.mp4) file. Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.molstruc.2026.147607](https://doi.org/10.1016/j.molstruc.2026.147607).

Data availability

Data will be made available on request.

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