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# Optimizing the aryl-triazole of cjoc42 for enhanced gankyrin binding and anti-cancer activity

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ABSTRACT

Gankyrin is an oncoprotein overexpressed in numerous cancer types and appears to play a key role in regulating cell proliferation, cell growth, and cell migration. These roles are largely due to gankyrin's protein-protein interaction with the 26S proteasome. We previously published a study exploring the aryl sulfonate ester of cjoc42 in an effort to enhance gankyrin binding and inhibit cancer cell proliferation. In order to further improve the gankyrin binding ability of the cjoc42 scaffold, an extensive SAR for the aryl-triazole moiety of cjoc42 was developed. Our cjoc42 derivatives exhibited enhanced gankyrin binding, as well as enhanced antiproliferative activity against Hep3B, HepG2, A549, and MDA-MB-231 cancer cell lines.

Gankyrin is a key protein for regulating numerous oncogenic and inflammatory pathways through its various protein-protein interactions (PPIs).[1–5] Additionally, gankyrin plays an integral role in signal transduction and its overexpression is associated with poor prognosis as well as aggressive proliferation in hepatocellular carcinoma, breast cancer and lung cancer.[1,6–13] Despite its prominent role in the onset and progression of these cancers, to date there is no small molecule inhibitor of gankyrin which is a viable drug candidate. Therefore, there is a need to develop novel inhibitors of gankyrin to explore their potential as anticancer therapeutics.

A number of protein-protein interactions (PPIs) involving gankyrin are responsible for the degradation of tumor suppressor proteins and the subsequent development of certain cancers. For example, gankyrin aids in the hyperphosphorylation of retinoblastoma protein (pRb) by binding to cyclin-dependent kinase 4 (CDK4), resulting in the proteasomal degradation of pRb. In a similar manner, gankyrin binds the ubiquitin ligase MDM2, increasing its ubiquitination of p53, and leading to the subsequent degradation of p53 by the proteasome.[2,14–17] Additionally, gankyrin is one of a number of repeat-protein "chaperones" required for the proper assembly of the proteasome.[3,18,19] It binds the S6 ATPase subunit of the 19S regulatory cap, which in turn enhances the targeting of p53, pRb, and other tumor suppressor proteins to the proteasome for degradation, illustrating gankyrin's prominent role in the degradation of important tumor suppressor proteins (TSPs).[2,3,20] Gankyrin is also responsible for the degradation of other TSPs, such as: CCAAT/enhancer binding protein α (C/EBPα), RNA CUG triplet repeats binding protein 1 (CUGBP1), and hepatocyte nuclear factor 4α (HNF4α).[19,21–23] Reduced levels of TSPs due to gankyrin overexpression results in the onset and progression of multiple cancer types (e.g., lung, breast and liver). It has been shown that preventing the degradation of TSPs by inhibiting specific PPIs results in decreased cell growth and decreased cancer cell migration. Furthermore, decreased levels of gankyrin, and its related PPIs, has also been associated with decreased cancer cell growth and migration, illustrating its potential as a therapeutic target.[5,16,24,25]

The first small molecule inhibitor of the gankyrin-S6 ATPase interaction (cjoc42) was recently discovered in 2016.[26] A subsequent study demonstrated that cjoc42 can inhibit the proliferation of certain liver cancer cell lines (huh6 and hepa1c1c7).[21] Our previous work establishing an SAR for the aryl sulfonate ester of cjoc42 identified multiple derivatives with enhanced gankyrin binding (higher $\Delta T_m$), as well as improved anti-cancer activity against Hep3B and HepG2 cells as compared to cjoc42 (Fig. 1, AFM-1-2).[27] Therefore, we sought to explore other structural features of cjoc42 to further shed light on the required structural features for binding gankyrin while also seeking to identify molecules with enhanced anti-cancer activity as compared to cjoc42.

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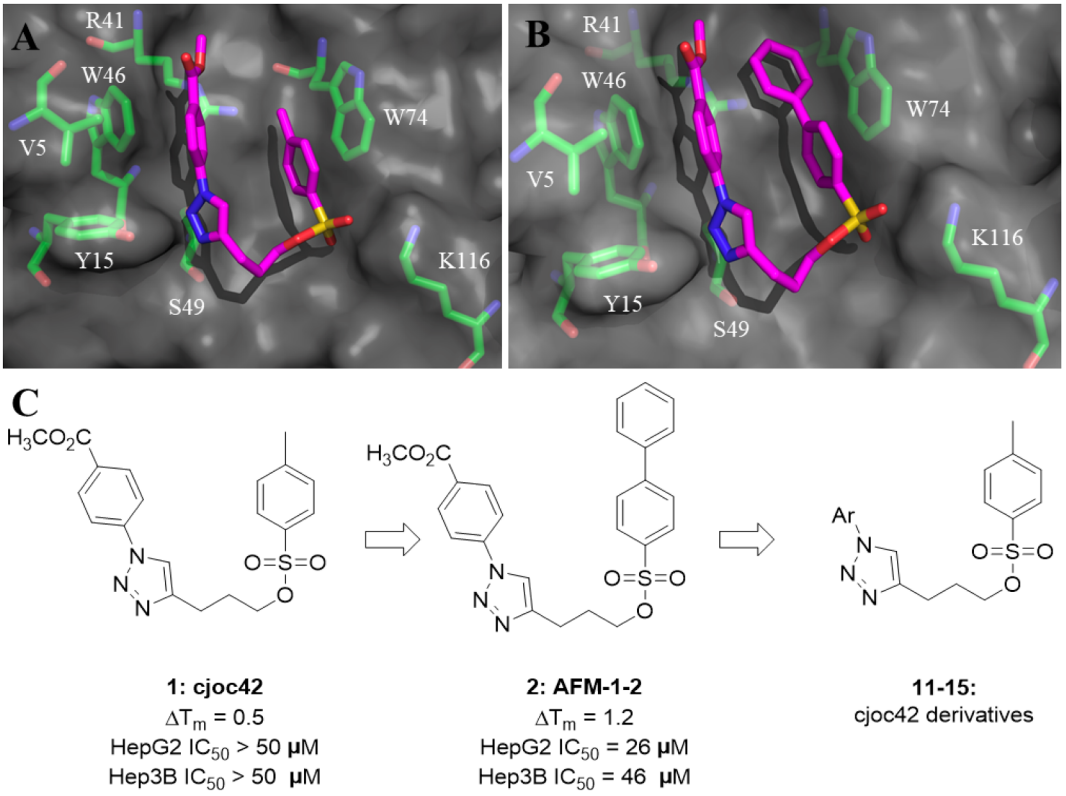
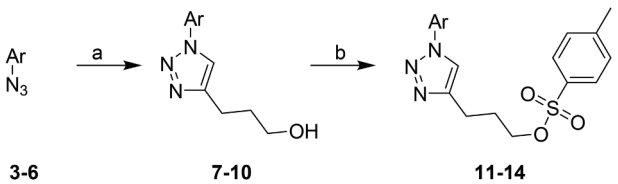



**Fig. 1.** Molecular modeling of cjoc42 (**1**) and its derivative AFM-1-2 (**2**). (A) Cjoc42 (**1**) docked to its proposed gankyrin binding site (PDB: 1QYM). (B) AFM-1-2 (**2**) docked to its proposed gankyrin binding site (PDB: 1QYM). (C) Structure of cjoc42 (**1**), AFM-1-2 (**2**) and targeted cjoc42 derivatives **11–15** (Ar = substituted aryl group).

Cjoc42's ability to bind gankyrin appears to be due to interactions with 5 key amino acid residues (Y15, R41, W46, S49, W74, and K116) as suggested by its proposed binding (Fig. 1A). From this, it appears that the methyl 4-azidobenzoate group of cjoc42 is engaged in a π-π interaction with W46 and a possible hydrogen bonding interaction with R41.[26] Utilizing this information, we sought to establish an SAR for this aryl ring in an effort to improve gankyrin binding. Therefore, an extensive SAR was established by substituting a variety of different functional groups (Fig. 1C, Ar substitutions) at the 2-, 3-, and 4-positions of the corresponding phenyl ring, as well utilize various heteroaromatic systems. The substitutions were made to optimize the π-π interaction of the phenyl ring with the indole ring of W46, optimize a possible hydrogen bonding interaction with R41, as well as probe for potentially new interactions.

Cjoc42 derivatives **11–14** were synthesized (Scheme 1) with the goal of substituting the 4-methyl ester group of cjoc42 with different functional groups as well as altering the position to the *ortho*, *meta* and *para* positions on the phenyl ring linked to the triazole ring. The synthesis of cjoc42 derivatives **11–14** (Scheme 1) commenced with either the diazotization of a series of anilines followed by the addition of sodium azide to generate a series of substituted azido intermediates, or by the nucleophilic substitution of an aryl halide with sodium azide




**Scheme 1. Reagents:** (a) 4-pentyn-1-ol, sodium ascorbate, $CuSO_4$, THF/$^t$BuOH/$H_2O$; (b) *p*-toluenesulfonyl chloride, TEA, DMAP, $CH_2Cl_2$. Ar = substituted aryl group.

to generate azido intermediates **6a** and **6c**. These azido intermediates then underwent a copper-catalyzed alkyne-azide cycloaddition (CuAAc) with 4-pentyn-1-ol in the presence of sodium ascorbate and copper sulfate to afford triazole intermediates **7–10** in modest to good yields over 2 steps (41–76%). Triazole intermediates **7–10** then underwent a nucleophilic substitution reaction with *para*-toluenesulfonyl chloride in the presence of triethylamine and 4-dimethylaminopyridine to generate desired cjoc42 derivatives **11–14** in modest to good yield (42–82%).

The relative ability of cjoc42 derivatives **11–15** to bind gankyrin was determined via a protein thermal shift assay. A protein thermal shift assay determines the temperature at which a protein denatures ($T_m$). When a ligand effectively binds a protein, the $T_m$ value shifts in comparison to the protein without a bound ligand. This change in $T_m$ value is then represented as the $\Delta T_m$ value or protein thermal shift, where the larger the $\Delta T_m$, the more effectively the ligand binds the protein of interest. Therefore, in order to evaluate the relative binding affinities of our cjoc42 derivatives to gankyrin, the differences in $T_m$ values ($\Delta T_m$) were determined. This provided a method for ranking cjoc42 derivatives **11–15** as well as compare them to cjoc42. The $\Delta T_m$ values obtained from thermal shift evaluation of cjoc42 derivatives **11–15** are shown in Fig. 2–5.

Cjoc42 derivatives **11a-11l** (Fig. 2) presented multiple derivatives with increased $\Delta T_m$ values as compared to cjoc42. Substitution of the methyl ester of cjoc42 with a simple methyl group (**11a**) resulted in a 3-fold increase in $\Delta T_m$. However, increasing the functional group size at the 4-position with 4-Et (**11b**) and 4-$^i$Pr (**11c**) groups was met with reduced $\Delta T_m$ values as compared to their 4-Me counterpart (**11a**). Furthermore, substitutions with other bulky groups such as 4-Ph (**11d**) and 4-I (**11j**) demonstrated a complete loss in binding affinity. Interestingly, large groups with increased electron withdrawing potential, such as 4-$CF_3$ (**11e**), 4-Cl (**11h**), and 4-Br (**11i**), exhibited significantly increased $\Delta T_m$ values as compared to cjoc42. However, smaller electron withdrawing groups such as 4-F (**11g**) and 4-CN (**11k**) did not exhibit

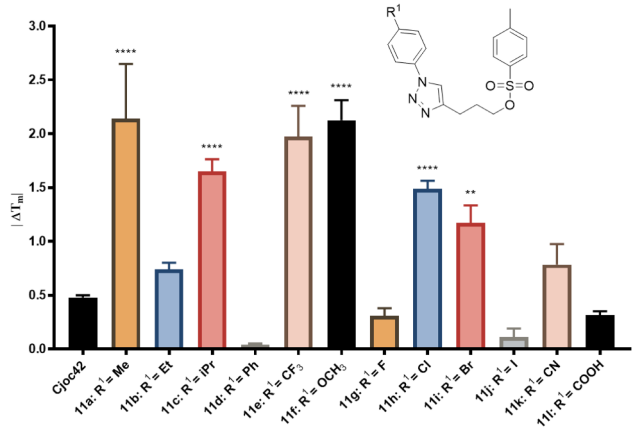



**Fig. 2.** Evaluation of cjoc42 derivatives **11a-11l** for relative gankyrin binding. Compounds **11a-11l** (300 μM) and gankyrin (20 μM) were incubated together in a thermal shift assay and their respective $\Delta T_m$ values were determined. DMSO (5%) served as a negative control (vehicle), and cjoc42 (300 μM) served as a positive control. All compounds were tested in triplicate. $^{**}0.001 < p < 0.01$, $^{***}0.0001 < p < 0.001$.

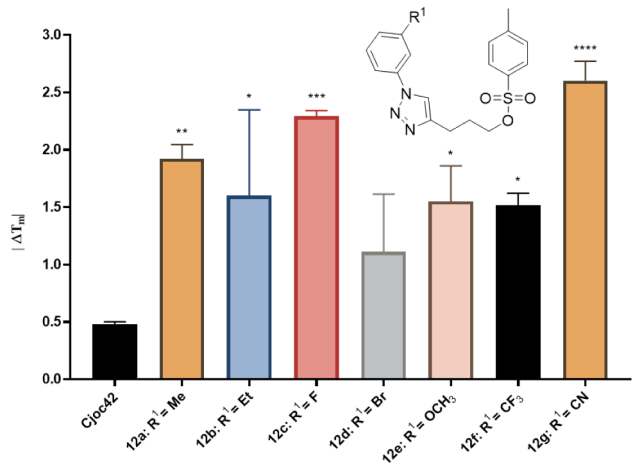



**Fig. 3.** Evaluation of cjoc42 derivatives **12a-12g** for relative gankyrin binding. Compounds **12a-12g** (300 μM) and gankyrin (20 μM) were incubated together in a thermal shift assay and their respective $\Delta T_m$ values were determined. DMSO (5%) served as a negative control (vehicle), and cjoc42 (300 μM) served as a positive control. All compounds were tested in triplicate. $^{*}0.01 < p < 0.05$, $^{**}0.001 < p < 0.01$, $^{***}0.0001 < p < 0.001$, $^{****}0.00001 < p < 0.0001$.

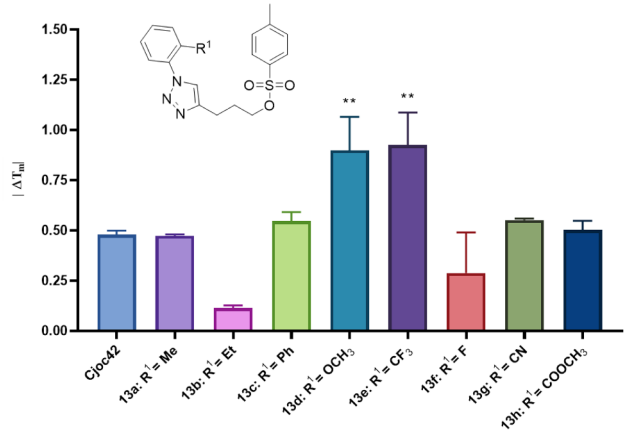



**Fig. 4.** Evaluation of cjoc42 derivatives **13a-13h** for relative gankyrin binding. Compounds **13a-13h** (300 μM) and gankyrin (20 μM) were incubated together in a thermal shift assay and their respective $\Delta T_m$ values were determined. DMSO (5%) served as a negative control (vehicle), and cjoc42 (300 μM) served as a positive control. All compounds were tested in triplicate. $^{**}0.001 < p < 0.01$.

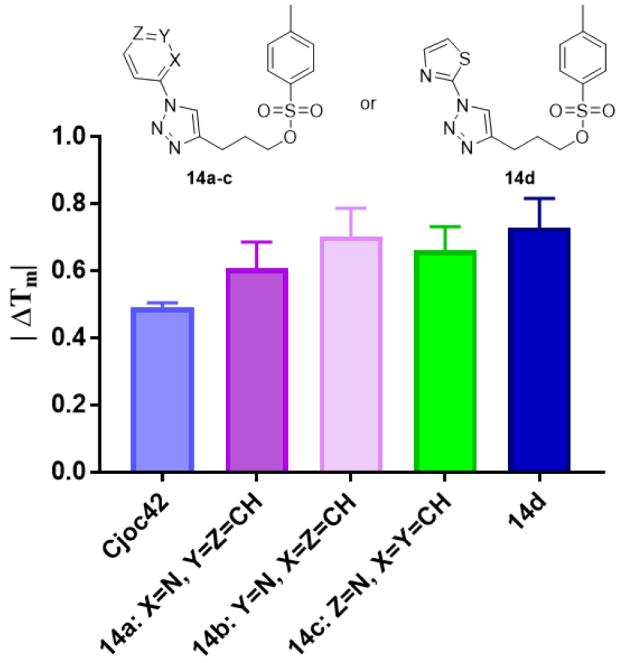
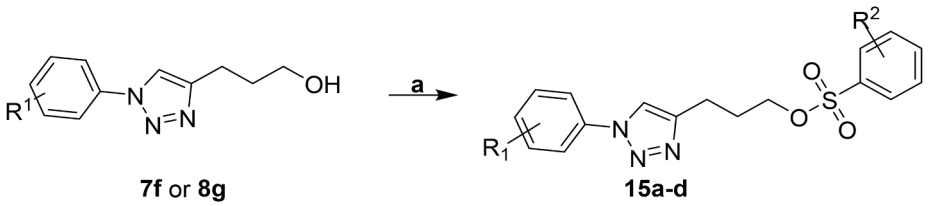



**Fig. 5.** Evaluation of cjoc42 derivatives **14a-14d** for relative gankyrin binding. Compounds **14a-14d** (300 μM) and gankyrin (20 μM) were incubated together in a thermal shift assay and their respective $\Delta T_m$ values were determined. DMSO (5%) served as a negative control (vehicle), and cjoc42 (300 μM) served as a positive control. All compounds were tested in triplicate.

any significant enhancement in $\Delta T_m$. The electron donating and potential hydrogen bond accepting 4-OMe group (**11f**) did exhibit a substantial increase in $\Delta T_m$, suggesting that similar groups should also be explored at this position in the future. The enhanced binding of 4-OMe may be due to a gained hydrogen bonding interaction with D39 (Fig. 7) as well as an optimized π-π interaction with W46 (Fig. 7).

When our focus moved to the 3-position (Fig. 3), the gankyrin binding affinity relative to cjoc42 was increased for all derivatives (**12a**-**12g**). At this position, small electron withdrawing groups displayed the greatest increases in $\Delta T_m$, with 3-F (**12c**) and 3-CN (**12h**) exhibiting 5-fold and 6-fold increases in $\Delta T_m$, respectively, as compared to cjoc42. This enhanced binding may be due to a gained hydrogen bonding interaction with R41 (Fig. 7) as well as an optimized π-π interaction with W46. These 2 compounds also exhibit the highest $\Delta T_m$ values for gankyrin to date.

Substitutions at the 2-position (Fig. 4, compounds **13a**-**13h**) only resulted in 2 derivatives (**13d** and **13e**) with improved gankyrin binding relative to cjoc42. While 2-OMe (**13d**) is electron donating and 2-$CF_3$ (**13e**) is electron withdrawing, they resulted in similar $\Delta T_m$ values. This suggests that sterics may be a significant factor in binding. This was further supported by 2-Me (**13a**) and 2-Ph (**13c**) exhibiting similar gankyrin binding to cjoc42, while 2-Et (**13b**) demonstrated a substantial decrease in relative binding affinity. It was also found that other electron withdrawing groups such as 2-F (**13f**), 2-CN (**13g**), and 2-$CO_2$Me (**13h**) exhibited decreased gankyrin binding affinities as compared to cjoc42. The enhanced binding affinity of 2-$CF_3$ (**13e**) may be due to a gained hydrophobic interaction of the trifluoromethyl group




**Scheme 2. Reagents:** (a) Biphenyl-4-sulfonyl chloride or 4-iodobenzenesulfonyl chloride, DMAP, TEA, $CH_2Cl_2$.

with I79 (Fig. 7) as well as an optimized π-π interaction with W46 (Fig. 7).

We next turned our attention to substituting the phenyl ring attached to the triazole with various heteroaromatic systems. Compounds **14a-d** demonstrated modest but not significant improvements in gankyrin binding as compared to cjoc42. Substituting the phenyl ring of cjoc42 with the 2- and 4-pyridine rings (**14a and 14c**, respectively) exhibited similar gankyrin binding affinities, whereas 3-pyridine (**14b**) displayed a slightly better binding affinity than cjoc42, **14a**, and **14c**. Substituting the phenyl ring with a thiazole (**14d**) ring also resulted in a modest but not significant improvement in gankyrin binding affinity as compared to cjoc42. Since compounds **14a-d** did not show a significant improvement in gankyrin binding affinity as compared to cjoc42, they were not further evaluated in cell-based assays.

As we previously reported, substituting the *p*-tosyl group of cjoc42 with a biphenyl-4-sulfonate ester group or 4-iodobenzene sulfonate ester group resulted in a ~2-fold improvement in gankyrin binding (Fig. 1, **AFM-1-2** and **JA-1-38**, respectively). Armed with this knowledge, we sought to combine these features with our most potent aryltriazole substitutions. Therefore, we synthesized cjoc42 derivatives **15a-d** to combine the best features of the $R^1$ and $R^2$ substitutions to cjoc42 (Scheme 2). The synthesis relied on constructing key triazole intermediates **7–9** as previously described. Intermediates **7–9** then underwent a nucleophilic substitution with biphenyl-4-sulfonyl chloride or 4-iodobenzenesulfonyl chloride to afford compounds **15a-d** in good yield (57–71%).

Cjoc42 derivatives **15a-d** presented only 2 derivatives, **15a** and **15d**, which exhibited improved gankyrin binding affinity as compared to cjoc42 (Fig. 6). However, all four derivatives demonstrated decreased $\Delta T_m$ values as compared to **AFM-1-2**, **JA-1-38**, **11f**, and **12e** ($\Delta T_m$ < 1.0). Although disappointing, this loss in binding affinity

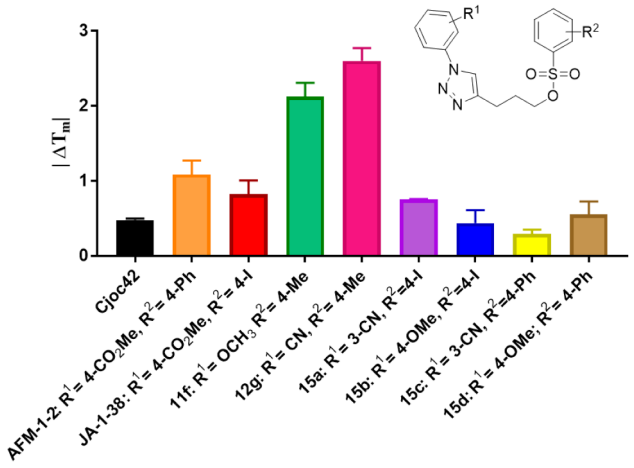



**Fig. 6.** Evaluation of cjoc42 derivatives **15a-15d** for relative gankyrin binding. Compounds **15a-15d** (300 μM) and gankyrin (20 μM) were incubated together in a thermal shift assay and their respective $\Delta T_m$ values were determined. DMSO (5%) served as a negative control (vehicle), and cjoc42 (300 μM) served as a positive control. All compounds were tested in triplicate.

suggests a change in binding mode causing each aryl substitution to no longer optimally interact with gankyrin. Future derivatives will utilize these findings to help guide our design.

The 5 most potent binders of gankyrin (**11a**, **11e**, **11f**, **12c**, and **12g**) had their anti-proliferative activity assessed in four gankyrin-overexpressing cancer cell lines (Table 1, HepG2, Hep3B, A549, and MDA-MB-231).[10,28–30] Cjoc42 did not exhibit any ability to inhibit proliferation in these four cell lines when evaluated up to 100 μM. However, all 5 cjoc42 derivatives (**11a**, **11e**, **11f**, **12c**, and **12g**) demonstrated an ability to inhibit cell proliferation at 100 μM, resulting in $IC_{50}$ values less than 100 μM. Specifically, **11a** exhibited the greatest anti-proliferative effect in MDA-MB-231 cells with an $IC_{50}$ value of 36.6 μM. Compound **11e** demonstrated the greatest ability to inhibit proliferation in both HepG2 and Hep3B cells with $IC_{50}$ values of 42.6 μM and 42.7 μM, respectively. Compound **11f** then displayed the best ability to inhibit A549 cell proliferation resulting in an $IC_{50}$ value of 74.6 μM. While compounds **12c** and **12 g** also exhibited an ability to inhibit Hep3B and HepG2 cells, neither proved more potent than compound **11e**. Interestingly, compounds **11a**, **11e**, **11f**, **12c**, and **12 g** did demonstrate similar anti-proliferative activity to cjoc42 in certain cell lines despite their enhanced gankyrin binding ability. Specifically, each of these compounds exhibited an $IC_{50}$ value greater than 100 μM in at least one of the cell lines they were evaluated in (Table 1). Although the reason for this is currently unknown, it could be due to poor membrane permeability and/or a lack of metabolic stability (i.e., sulfonate ester) and will be the focus of future studies.

In summary, a novel series of cjoc42 derivatives with a modified Ar group (Fig. 1) were designed, synthesized, and evaluated for their ability to bind gankyrin and inhibit liver, lung and breast cancer cell proliferation. Modifications to the aryl triazole moiety resulted in 3 new derivatives of cjoc42 (**11f**, **12g**, and **13e**) with superior anti-cancer activity in Hep3B, HepG2, A549, and MDA-MB-231 as compared to cjoc42. In terms of gankyrin binding, an extensive SAR (Fig. 8) revealed that electron donating substituents were favored at the R = 4-position (**11e**), while at the R = 3-position the electron withdrawing CN group was favored (**12g**), and at the R = 2-position the electron withdrawing $CF_3$ group was preferred (**13e**). These second generation cjoc42 derivatives also exhibited enhanced gankyrin binding compared to **AFM-1-2** (Fig. 1), our most potent first generation cjoc42 derivative.[27] However, **AFM-1-2** demonstrated similar or superior anti-proliferative activity against Hep3B and HepG2 cells than our most potent gankyrin-binding second generation cjoc42 derivatives **11a**, **11e**, **11f**, **12c**, and **12g**. In addition, second generation cjoc42 derivatives **11f**, **12g**, and **13e** all exhibited improved anti-proliferative activity against Hep3B, HepG2, A549 and MDA-MB-231 cells compared to cjoc42. These findings mark a significant advance over cjoc42 in terms of both gankyrin binding as well as anti-cancer activity in liver, lung, and breast cancers.

## 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.

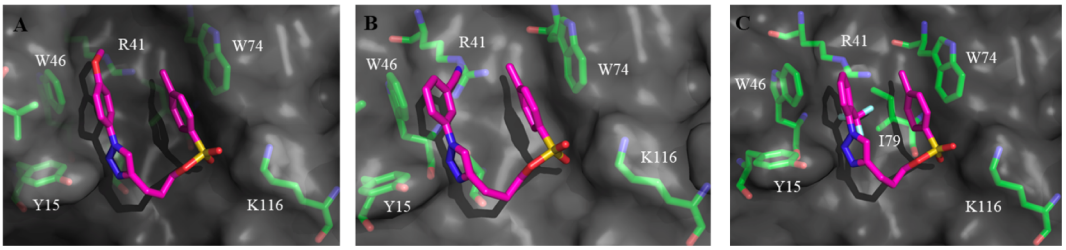



**Fig. 7.** Predicted binding of cjoc42 derivatives **11f** (**A**), **12g** (**B**), and **13e** (**C**) to gankyrin based on *in silico* docking studies (PDB: 1QYM).

**Table 1**
Anti-proliferation evaluation of cjoc42 and derivatives **11a**, **11e**, **12c**, **12e**, and **12 g** in Hep3B, HepG2, A549 and MDA-MB-231 cells.[a–e]

| Compound | Hep3B $IC_{50}$ (μM) | HepG2 $IC_{50}$ (μM) | A549 $IC_{50}$ (μM) | MDA-MB-231 $IC_{50}$ (μM) |
|---|---|---|---|---|
| **cjoc42** | > 100 | > 100 | > 100 | > 100 |
| **AFM-1–2** | 26.0 ( ± 3.5) | 46.2 ( ± 6.1) | NT | NT |
| **11a** | > 100 | > 100 | 79.9 ( ± 4.7) | 36.6 ( ± 5.5) |
| **11e** | 42.7 ( ± 5.8) | 42.6 ( ± 6.5) | > 100 | > 100 |
| **11f** | > 100 | > 100 | 74.6 ( ± 4.4) | 72.8 ( ± 7.1) |
| **12c** | 82.1 ( ± 16.0) | 61.8 ( ± 12.1) | > 100 | > 100 |
| **12g** | 44.9 ( ± 8.5) | > 100 | > 100 | > 100 |

[a]Hep3B, HepG2, A549 and MDA-MB-231 cells were incubated for 24 h prior to drug addition. [b]Hep3B and HepG2 cells were incubated for 72 h at 37 °C in 5% $CO_2$ with the respective drug. A549 and MDA-MB-231 cells were incubated for 48 h at 37 °C in 5% $CO_2$ with the respective drug. [c]Cell proliferation was determined using an MTT assay. [d]All experiments were performed in replicates of 6–8. NT = not tested.

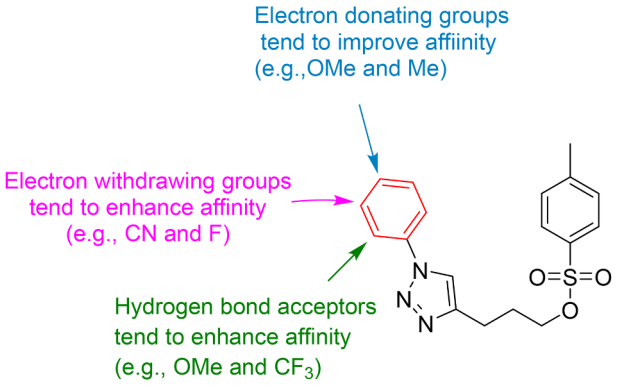



**Fig. 8.** Summary of aryl-triazole SAR.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127372.

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