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Developing structure–activity relationships from an HTS hit for inhibition of the Cks1–Skp2 protein–protein interaction

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ABSTRACT

Structure–activity relationships have been developed around 5-bromo-8-toluylsulfonamidoquinoline **1** a hit compound in an assay for the interaction of the E3 ligase Skp2 with Cks1, part of the SCF ligase complex. Disruption of this protein–protein interaction results in higher levels of CDK inhibitor p27, which can act as a tumor suppressor. The results of the SAR developed highlight the relationship between the sulfonamide and quinoline nitrogen, while also suggesting that an aryl substituent at the 5-position of the quinoline ring contributes to the potency in the interaction assay. Compounds showing potency in the interaction assay result in greater levels of p27 and have been shown to inhibit cell growth of two p27 sensitive tumor cell lines.

Ubiquitination of a protein provides a signal for its targeted degradation and recycling via the ubiquitin–proteasome pathway.[1–3] The process of ubiquitination takes place in a series of steps, beginning with the activation of ubiquitin through a ubiquitin-activating enzyme E1 followed by transfer to a ubiquitin-conjugating enzyme E2. Finally the ubiquitin is linked to the lysine of the target protein in the presence of an ubiquitin-protein ligase E3 (referred to as a ubiquitin ligase). Chains of four or more ubiquitin domains activates the degradation process by the proteasome.

The E3 ubiquitin ligase acts as a substrate recognition module for the ubiquitination system in which each E3 provides specificity for only a small number of substrates. This specificity makes E3 ligases attractive targets for drug discovery (analogous to kinases), for instance by preventing degradation of pro-apoptotic proteins in cancer cells.[4] Indeed the proteasome inhibitor Bortezomib (marketed as Velcade®) is indicated for the treatment of multiple myeloma and is thought to be preventing degradation of pro-apoptotic proteins.[5] There is now a second class of proteasome inhibitors based on the natural product epoxomicin—the recently approved Carfilzomib (marketed as Kyprolis®) and Oprozomib, which is still in clinical trials.

The development of small molecule E3 ligase inhibitors is challenging due to the requirement of the molecules to disrupt protein–protein interactions (PPI's). PPI's are an area that has not been well explored in small-molecule drug-discovery since the interaction surfaces are often large with flat or shallow grooves at the interfaces.[6] This is in contrast to the tight, well defined pockets present in traditional enzymes or receptors. However, the discovery of molecules targeting PPI's has gained recognition and provides potential for novel treatments directed at significant human maladies.[7,8] Indeed there have been several examples of groups studying the disruption of E3 ligase binding. One of the initial investigations in this area came from the Roche group, working on the disruption of binding between p53 and MDM2. MDM2 serves as the E3 ligase for p53 promoting degradation. The work resulted in the identification of *cis*-imidazolines known as Nutlins, which displace p53 from its complex with MDM2 in the 100–300 nM range.[9] These efforts have spurred a number of groups to develop structure–activity relationships (SAR) around these and similar structures, resulting in compounds that inhibit the p53–MDM2 interaction with single digit nanomolar potencies and below.[10–12] Further there have been several reports of small molecules being used to target E3 ligases including the von Hippel-Lindau ligase to disrupt the VHL–HIF-1α interaction[13] as well as a non-ligase PPI between HIF1α and HIF1β.[14]

The Skp1-Cullin 1-F-Box (SCF) family of E3 ligases are a well characterized family held together through PPI's. The complex consists of the scaffold protein Cullin-1, which binds Roc1 (recruiting the E2) and Skp1 (recruiting the F-Box protein). For this study we

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were interested in the E3 ligase complex responsible for p27, the substrate recognition component Skp2 and an adaptor protein Cks1 (Fig. 1). p27, a CDK inhibitor, is a negative regulator of cell cycle progression. Low levels of p27 have been implicated in a number of cancers,[15] while elevated levels of Cks1 have been associated with low levels of p27 and poor prognosis in cancer patients.[16,17] To our knowledge there have not been any reports of compounds targeted to the Cks1–Skp2 PPI; however, there have been several groups that have targeted the SCF ligases with the goal of increasing levels of p27. Molecules have been identified disrupting PPI's between Skp1 and Skp2[18] and Skp2–Cks1–p27.[19] Further there have been several reports of molecules that interact with alternative E3 ligases in the SCF system including Cdc4,[20] Met30[21] and βTRCP1.[22] The inhibitors are in the 1–10 μM range and were identified through the screening of libraries with little SAR maturation.

Our study began by developing a high through-put screen (HTS) for inhibitors of the Cks1–Skp2 PPI. From the screen a number of hit compounds were identified representing a range of structurally diverse scaffolds. One intriguing hit was the sulfonamidoquinoline **1** (Fig. 1), a sub-micromolar inhibitor of the Cks1–Skp2 PPI. Support for inhibition of this interaction was observed in cells expressing a GFP tagged p27, where we were able to see an increase in p27 GFP both on Western blot and using an ELISA format, suggesting that in a cellular system the compound indeed inhibits the ubiquitination and degradation of p27 (Fig. 1 and Supplementary Material). A similar effect was also observed with compound **2** (Table 1).

With a hit compound in hand our investigations began by probing the relationship between the quinoline nitrogen and the sulfonamide linkage using compounds available in our compound library. The corresponding amide, naphthalene, *N*-methylsulfonamide, isoquinoline and reversed sulfonamide (**3**–**7**) were investigated, all of which were inactive in the interaction assay suggesting that the relationship between the quinoline and sulfonamide was important for potency (Fig. 1).

Table 1
Potency of compounds **1**, **2**, **8**–**18** in the interaction assay and their ability to increase p27-GFP

Compounds	Cks1–Skp2 (μM)	GFP-p27
1	0.84	+
2	2.18	+
8	0.54	+
9	0.55	+
10	1.73	+
11	5.54	+
12	7.84	+
13	Inactive	–
14	Inactive	–
15	Inactive	–
16	3.57	+
17	1.15	–
18	1.74	NA

The bromo group on the quinoline of **1** was an ideal handle for the introduction of a range of substituents through cross-coupling chemistries. The Suzuki reaction yielded a series of biaryl compounds (**8**–**12**) while Buchwald–Hartwig reaction allowed for the introduction of cyclic amines (**13**–**15**) (Scheme 1).

Compounds **8**–**15** were screened in the interaction assay and those compounds that showed promising potency were further screened in the p27-GFP assay (Table 1). Both the phenyl **8** and the furanyl **9** derivatives showed improved potency over the hit compound **1** (in the 500 nM range). Both compounds also increase the levels of p27 in the p27-GFP assay suggesting that the molecules are indeed inhibiting the SCF^{Skp2} ligase complex in cells.

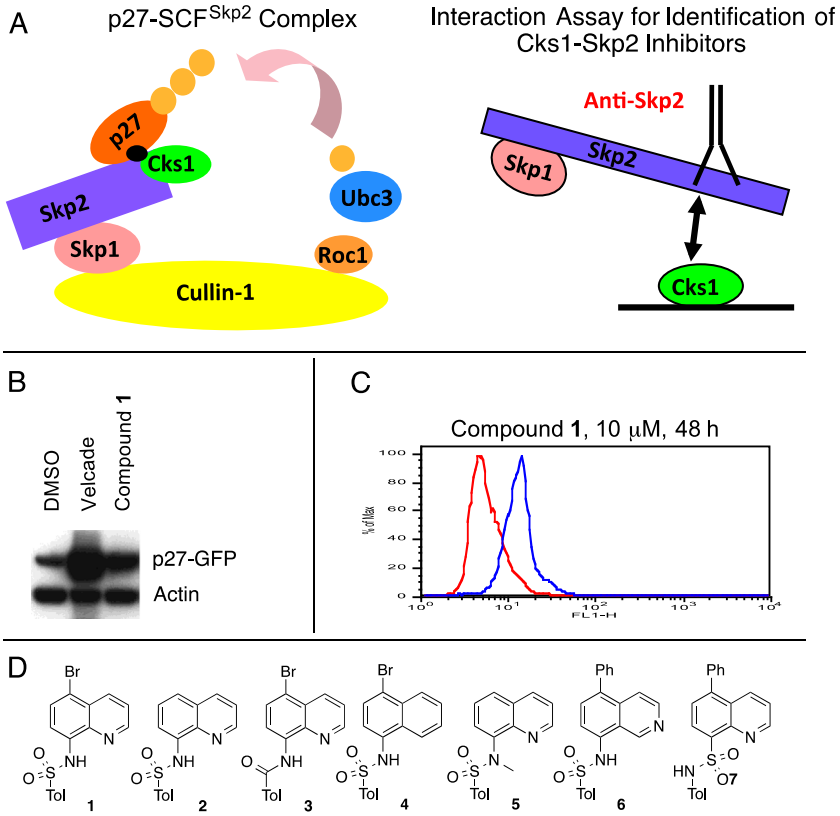

Figure 1. (A) Schematic representation of the p27-SCF^{Skp2} complex and the assay to investigate inhibitors of the Cks1–Skp2 interaction, (B) Western blot showing increase in p27-GFP in presence of Velcade or **1**, (C) p27-GFP assay in ELISA format showing the increase in p27-GFP with compound (blue line) relative to DMSO (red line), (D) hit compound **1** and analogues to probe the relationship between the sulfonamide and the quinoline.

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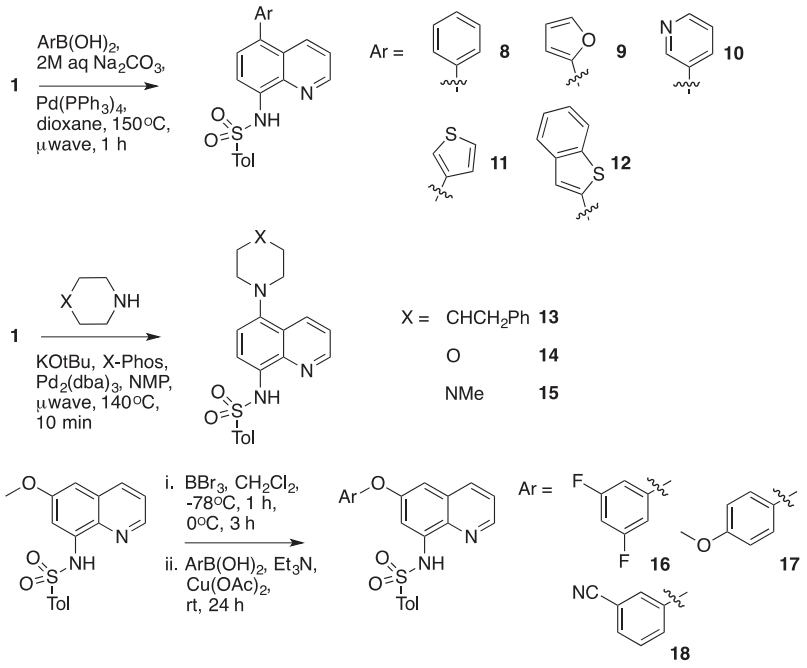
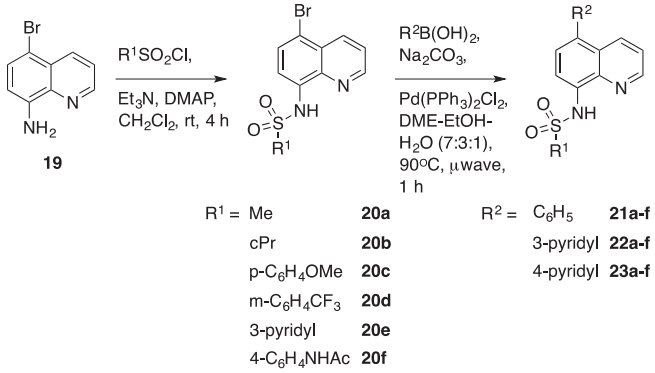


Scheme 1. Synthesis of 5-aryl, 5-amino- and 6-*O*-arylquinoline compounds.

The pyridyl compound **10** is also potent in the interaction assay, while the thiophene **11** and benzothiophene **12** compounds are less potent. Interestingly, despite the weaker potency of compounds **11** and **12** in the interaction assay both raised levels of the p27 in the p27-GFP assay as did the pyridyl compound **10** (see Supplementary Material). By contrast, the benzylpiperidinyl, morpholino and *N*-methylpiperazinyl analogues **13**–**15** were inactive in both the interaction and p27-GFP assays. Moving the aryl group in **8** to both the 4- or 6-position of the quinoline ring resulted in compounds that showed weak or no activity in the interaction assay (see Supplementary Material for a list of compounds). However, the 6-*O*-(phenyl)quinolyl ethers **16**–**18** synthesized by Evans–Chan–Lam reaction between the corresponding arylboronic acid and 6-hydroxyquinoline resulted in a compounds that were able to inhibit the Cks1–Skp2 interaction (Scheme 1).[23–25]

Finally we examined the nature of the sulfonamido group. Beginning from the 8-amino-5-bromoquinoline **19** a series of sulfonamides **20a**–**20f** were generated from the corresponding sulfonyl chlorides. In each series a group of three Suzuki reactions was carried out using the phenyl, 3- and 4-pyridylboronic acids to generate a 6 × 3 matrix of compounds (Scheme 2). The sulfonyl group was varied to investigate both alkyl groups (methyl, cyclopropyl), and aryl groups, with the aryl groups were chosen to explore the effect of electron-withdrawing and electron-donating substituents on inhibition of the Cks1–Skp2 interaction.



Scheme 2. Synthesis of compounds to probe the sulfonamido group.

The results showed that the aromatic group was required with the both the methyl and cyclopropyl compounds **21a**,**b**–**23a**,**b** showing weak activity in the interaction assay and did not increase p27-GFP. However, both the *p*-methoxysulfonamido **22c**, **23c** and in particular the *m*-trifluorophenylsulfonamido compound **22d** showed excellent potency, especially **22c** and **22d** the analogues with the 3-pyridyl group at the 5-position of the quinoline.[26] Further these compounds increased levels of p27-GFP. Indeed **22d** was the more potent compound in the interaction assay and one of the most potent inhibitors of an SCF ligase interaction reported (Table 2). The pyridylsulfonamido compounds **21e**–**23e** showed reasonable potency in the interaction assay, but mixed effects in p27-GFP levels. The acetamidosulfonamido compounds **21f**–**23f** were poor inhibitors. Taken as a whole the results suggest that the substituent on the sulfonamido group may extend into a hydrophobic region in the protein.

We were interested to know if the compounds were able to inhibit tumor cell growth. Thus, compounds that were able to inhibit the interaction of Cks1–Skp2 were screened in both the lung tumor cell line A549 and the fibrosarcoma HT1080 in a six-day cell proliferation assay. The cell lines have been shown to be sensitive to p27 (Table 3) both in the literature and in our own laboratories (see Supplementary Material).[27,28] The data was compared with

Table 2
Potency of the sulfonamide analogues in the Cks1–Skp2 interaction assay (μM), and their ability to increase p27-GFP in parentheses

	21	22	23
a	2.20 (+)	15.13 (−)	Inactive (−)
b	12.10 (−)	7.16 (−)	10.94 (−)
c	8.02 (+)	0.58 (+)	2.45 (+/−)
d	6.70 (+)	0.17 (+)	Not synthesized
e	2.20 (+)	1.67 (+/−)	1.21 (−)
f	7.60	14.49	6.55

Table 3
Potency of selected compounds against p27 sensitive cell lines in a six-day proliferation assay and in a two-day toxicity assay

	Interaction Cks1–Skp2	6-Day proliferation GI_{50} (μM)		2-Day proliferation IC_{50} (μM)	
	IC_{50} (μM)	A549	HT1080	H1299	A549
2	2.18	2.48	2.46	5.35	7.54
4	Inactive	48.5	27.36	Inactive	Inactive
8	0.54	2.36	1.64	8.56	7.22
9	0.55	26.0	9.78	Inactive	Inactive
10	1.73	6.02	4.22	6.95	7.13
11	5.54	2.36	0.64	4.09	4.63
12	7.84	0.39	0.15	Inactive	Inactive
16	3.57	4.11	0.08	2.46	2.94
17	1.15	4.09	0.74	11.34	16.92
18	1.74	6.48	6.83	Inactive	Inactive
22c	0.58	1.24	0.44	5.31	7.94
23c	2.45	3.06	1.32	3.90	5.91
22d	0.17	0.91	0.40	1.05	1.49
22e	1.67	5.24	1.07	Inactive	Inactive
23e	1.21	5.88	1.07	9.48	11.83

our standard two-day cell proliferation assay used to screen for toxicity in two cell lines—H1299 and the same A549 line.[29]

The results suggest that inhibition of the Cks1–Skp2 PPI results in inhibition of cell proliferation in p27 sensitive cell lines, with compounds exhibiting micromolar potencies. By contrast, compound **4**, which is inactive in the interaction assay is the weakest compound screened in the p27 sensitive six-day assay (IC_{50} >25 μM). The most potent compound in the interaction assay, the trifluoromethylsulfonamidoquinoline **22d**, shows sub-micromolar potency against both cell lines, which compares favorably with the compounds identified in previous studies targeting p27.[18,19] In the two-day assay the compound shows the most activity of those screened, however, **22d** is approximately two-fold more potent in the six-day assay. In general, the HT1080 cell line appears to be more sensitive with several compounds (**12**, **16**, **22c** and **22d**) showing sub-500 nM potency. An outlier is the benzothiophenequinoline **12**, a weak inhibitor in the PPI assay but the most potent compound against both cell lines in the six-day assay and inactive in the two-day assay. The cell data suggests the effect is p27-related, which is lent further credence by the ability of the compound to increase levels of p27-GFP in cells. It may require a more diverse set of compounds to fully interpret the data for compound **12**.

In summary we have shown that the ability of compounds to inhibit the Cks1–Skp2 PPI results in greater levels of p27 in cells, which in turn translates to effects on tumor cell growth. The SAR developed from the hit sulfonamidoquinoline **1**, demonstrates a key role for the sulfonamide NH and quinoline nitrogen for inhibition of the PPI. In addition, it appears that there are binding interactions that can be obtained by placing aromatic groups on the sulfonamide and at the 5-position of the quinoline ring. It is anticipated that developing further SAR will result in low nanomolar inhibitors of the Cks1–Skp2 PPI, analogous to the example of MDM2-p53.

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Supplementary data

A list of 4- and 6-substituted compounds screened, schemes for the synthesis of **6** and **7**, all experimental procedures including descriptions of the assays, data for the p27-GFP ELISA assay, a time course for proliferation of A549 cells in the presence of Skp2 siRNA and compound characterization.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.09.067. These data include MOL files and InChiKeys of the most important compounds described in this article.

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