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Identification of novel, selective and potent Chk2 inhibitors

Gary Larson, Shunqi Yan, Huanming Chen, Frank Rong, Zhi Hong and Jim Zhen Wu*

Drug Discovery, Valeant Pharmaceutical Research and Development 3300 Hyland Avenue, Costa Mesa, CA 92626, USA

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Abstract—A series of isothiazole carboxamidine compounds were synthesized and discovered as novel and selective inhibitors for Chk2. They are not active against the related Chk1 kinase. The structure-activity relationship studies were performed on the scaffold, and enzymatic kinetic analysis showed they are simple ATP competitive inhibitors with K_i values as low as 11 nM for Chk2. Computer modeling studies were employed to comprehend the mechanism of action and SAR of these compounds.

Genetic material is exposed to many environmental stresses such as radiation, ultraviolet light, and chemicals that could damage DNA. Unrepaired DNA damages in cells will accumulate erroneous genetic material and eventually lead to tumorigenesis or apoptosis. Without the activation of DNA damage-signaling transduction pathways and cell cycle checkpoints, cells would not have the chance to make the necessary repairs and maintain genetic stability.

Checkpoints are the fail-safes to ensure the cell cycle not to progress to the next stage until the previous step is completed.[1] The protein kinases, checkpoint kinase 1 (Chk1) and 2 (Chk2), are the major effectors of the replication checkpoint.[2] DNA damage can activate signal transduction pathways that lead to cell cycle arrest or apoptosis depending on the type and severity of the damage. This signaling network is initiated by the central sensors of DNA damage known as ATR and ATM protein kinases.[3] ATR generally responds to UV damage and chemical-induced stall of DNA replication forks, and subsequently promotes the degradation of cdc25 by activating Chk1. ATM similarly initiates the degradation of cdc25 but does so in response primarily to the double strand breaks in the DNA.[4] Activated ATM phosphorylates both Chk1 and Chk2, which in turn phosphorylate downstream cdc25A, cdc25C, BRCA1, and p53, and lead to cell cycle arrest at G1/S and G2/M checkpoints or apoptosis.

The crucial roles played by both Chk1 and Chk2 in mediating cellular responses to DNA damages implicate potential pharmacological value of their inhibitors in cancer therapy. Addition of a Chk1 inhibitor to a DNA damaging chemotherapy drug has been shown to sensitize tumor cells to chemotherapy and enhance drug's cytotoxic effect.[5] Conversely, selective inhibition of Chk2 has been reported to protect normal cells from DNA damage caused by ionizing radiation and enhance the efficacy of genotoxic agents in tumor cells. [5,6] Since nearly half of the cancer patients undergo radiotherapy and they all suffer from the side effects caused by radiation damage to the sensitive tissues, a small molecular Chk2 inhibitor may be beneficial in radiotherapy for an improved safety window.

To date, a few Chk inhibitors have been discovered and revealed in the literature. They include a broad spectrum protein kinase inhibitor staurosporine and its analogs such as UCN-01, Go6979, and isogranulatimide.[7] Selective Chk inhibitors are rare but have been reported. 4-(Aminoalkylamino)-3-benzimidazole quinolines are a series of specific Chk1 inhibitors.[8] A series of 2-arylbenzimidazole compounds were published recently as selective and potent inhibitors for Chk2.[9] By screening against a protein kinase target with our chemical library, we found a series of isothiazole carboxamidine compounds that exhibited potent protein kinase inhibitory activity.[10] Some of them possess potent selective inhibitory activity against Chk2 in the protein kinase specificity studies. Here, we describe the SAR of this series of compounds against Chk2, their enzymatic inhibition kinetics, and computer model analysis of their mode of action. A Chk2 enzymatic assay was developed to determine IC_{50} values of these compounds. The assay

Keywords: Protein kinase inhibitor; Chk2 inhibitor; Chk; Chk2; DNA damage.
* Corresponding author. Tel.: +1 714 545 0100x3024; fax: +1 714 668 3142; e-mail: jwu@valeant.com

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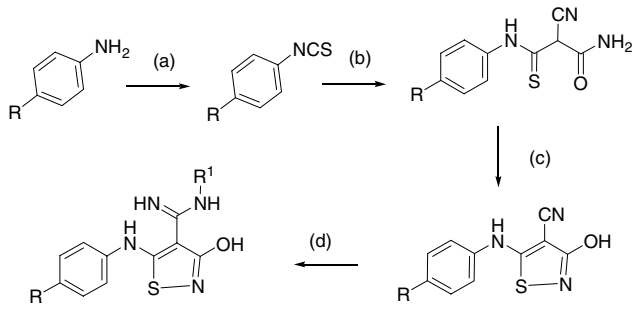


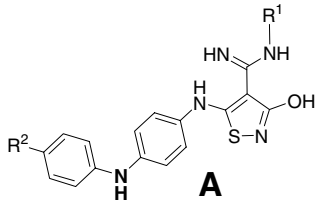
Scheme 1. Reagents and conditions: (a) $CSCl_2$, aq K_2CO_3, $CHCl_3$, rt, 2 h; (b) cyanoacetamide, KOH, DMF, rt, 16 h; (c) Br_2, EtOAc, rt, 1–2 h; (d) R^1–NH_2, EtOH, 90 °C, 16 h.

conditions are described in the notes.[14] The chemical synthesis of these compounds was based on the published protocol[10] and is outlined in Scheme 1.

The original hit against Chk2 from the protein kinase specificity analysis, 3-hydroxy-*N*-isopropyl-5-(4-phenoxy-phenylamino)isothiazole-4-carboximidamide (**16**), has an IC_{50} of 2.3 μM (Table 2). Preliminary SAR studies by replacing the bridge oxygen between two phenyl rings in **16** with an amine resulted in **7** with a 5-fold improvement of IC_{50} (Table 1). Further addition of a bromo to the *para* position of the left phenyl ring of **7** yielded **1** with an IC_{50} of 87 nM. Moreover the displacement of the carboxamidine by a cyano group totally abolished a compound's activity, underscoring the importance of carboxamidine as a part of key pharmacophore in the scaffold. These simple SAR exercises suggest that these compounds have tractable SAR, which prompted us to perform a more systematic SAR study of the scaffold. As summarized in Tables 1–3, three series of compounds, **A**, **B**, and **C**, were synthesized with modifications focused on the substitution R^1 and R^2, the linker X between two phenyl rings, and the left phenyl ring replacement. Their activities against Chk2 were determined.

As illustrated in Table 1, compounds in series **A** with a NH bridge linker between two phenyls consistently demonstrated higher inhibitory activity against Chk2 than

Table 1. The enzymatic IC_{50} values of series **A** compounds against Chk2 (in μM)



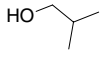
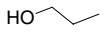
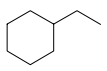
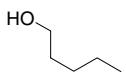
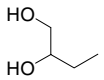
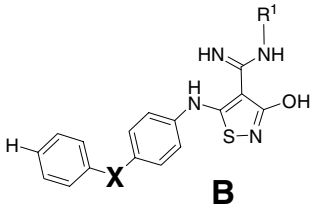
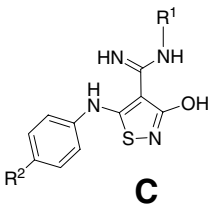
Compound	R^1	R^2	IC_{50}	Compound	R^2	IC_{50}
1		Br	0.087	**7**	H	0.42
2		Br	0.14	**8**	H	0.46
3		Br	0.22	**9**	H	1.0
4		Br	0.30	**10**	H	1.8
5		Br	0.31	**11**	H	1.2
6		Br	0.47	**12**	H	0.90
13		H	0.46			

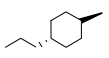
Table 2. The enzymatic IC_{50} values of series **B** compounds against Chk2 (in μM)



Compound	R^1	X	IC_{50}
14		–CH_2–	8.0
15		–N=N–	11
16		–O–	2.3
17		–SO_2–	>40
18		–CH_2–	37
19		–N=N–	11
20		–S–	19
21		–CO–	5.0

Table 3. The enzymatic IC_{50} values of series **C** compounds against Chk2 (in μM)



Compound	R^1	R^2	IC_{50}
22			19
23			40
24			>40
25			17

those in series **B**. Most of IC_{50} values in series **A** are under 1 μM, and they tolerate the different R^1 substitutions. For example, the addition of an extra hydroxyl group to R^1 slightly changes IC_{50} of **1** from 0.087 to 0.14 μM compared to **2**. Removal of one methyl group of R^1 from **2** only reduces 1.5-fold of potency in **3**. A longer and more flexible hydroxyl (**5**) or diol R^1 (**6**) lowers the potency by a few folds. Meanwhile, it seems that a more hydrophobic R^1 such as in **4** is tolerable but not favorable. On the other hand, IC_{50} values appear to be more sensitive to the substitution R^2 on the left side of the molecules. An unsubstituted R^2 (**7**) is 5-fold less potent than a bromo substituted **1**. Within the same series of compounds with $R^2 = H$ (**7**–**12**), their inhibitory activities range from 0.42 to 1.8 μM, and they are consistently 3- to 5-fold less active than their bromo substituted counterparts. Nevertheless, both R^1 and R^2 substitutions are relatively unimportant in determining their inhibitory activity against Chk2. The most potent compound from this series is **1**, which has an IC_{50} of 87 nM.

The effect of the linker atom X between the two phenyl rings was investigated in series **B**. It was found that any change from amine linker is detrimental to the activity (Table 2). A methylene linker in **14** and **18** resulted in a 20-fold loss of IC_{50} in comparison to the similar NH-linked **7** and **10**. Other linkers such as oxygen in **16**, thio in **20**, or –N=N– in **15** and **19** also yielded compounds with less potency. A sulfone substitution in **17** completely abolishes activity against Chk2.

We further assessed the importance of the left phenyl ring and the linker effect in the series **C** of compounds. As summarized in Table 3, a direct attachment of R^2 to the middle phenyl ring gave rise to either very weak or totally inactive compounds regardless of the nature of R^1 and R^2. For example, compounds **22** and **25** with a cyclohexyl linked directly to phenyl have IC_{50} values in double digit μM range. Compound **23** with R^2 of 1*H*-pyrazole is only marginally active. A combination of R^1 of 2-isopropyl-1-ol- and R^2 of 1*H* -pyrazole in **24** completely eliminates any activity against Chk2.

To understand the action mechanism of these inhibitors, we picked a representative compound **2** for Chk2 inhibition kinetic analysis. Using a radioactive-based filter binding assay [14] with the assay conditions highlighted in Figure 1, we determined this compound's inhibition kinetics as simple competitive with ATP (Fig. 1). The inhibition constant K_i was 11 nM. This result suggests that these compounds likely act by directly binding to the ATP site of Chk2. Because they are ATP competitive, the IC_{50} values reported here are highly influenced by the ATP concentration in the assay. In our assay, a relatively high concentration of ATP at 100 μM was used, in which the IC_{50} for **2** was 140 nM. Decreased ATP concentration of 10 μM resulted in a much lower IC_{50} of 43 nM. In a similar Chk1 enzymatic assay, the IC_{50} of **2** was more than 40 μM, suggesting that **2** is a selective Chk2 inhibitor. Moreover, we chose compound **8** for protein kinase inhibition specificity studies using Upstate's KinaseProfiler™ assays. Although it did exhibit some weak activity against two other protein kinases, the IC_{50}s are more than 20-fold higher than that for Chk2.

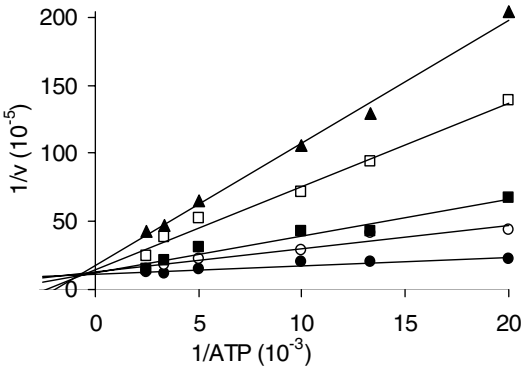


Figure 1. Lineweaver–Burk analysis of inhibition kinetics of **2** against Chk2. The study was performed at various concentrations of **2** (0 (●), 25 (○), 50 (■), 100 (□), and 200 (▲) nM) with ATP concentrations varied from 0 to 200 μM. The double reciprocal plots were fitted best to a simple competitive inhibition model.

To further comprehend the molecular mechanism of how these compounds inhibit Chk2 and why their activities differ so modestly for various R^1 and R^2 substituents but so radically for any change on the linker X, we performed computer modeling studies. As the previous kinetic analysis indicated, they are ATP competitive inhibitors and they likely bind directly to the ATP binding pocket of Chk2. Using a recently solved Chk2 co-crystal structure with a debromohymenialdisin inhibitor as a template,[13] compound **7** was docked into the ATP binding site of Chk2 (PDB code: 2CN8) using the Glide docking program.[12] All water molecules around the ATP site were removed and hydrogen atoms were added using Pprep of Glide. Grids for Glide docking were calculated using the bound ligand as the reference of binding site in the Chk2 protein. The Glide docking was performed using extra-precision mode with up to 10 poses saved for analysis. The top-scored pose is depicted in Figure 2.

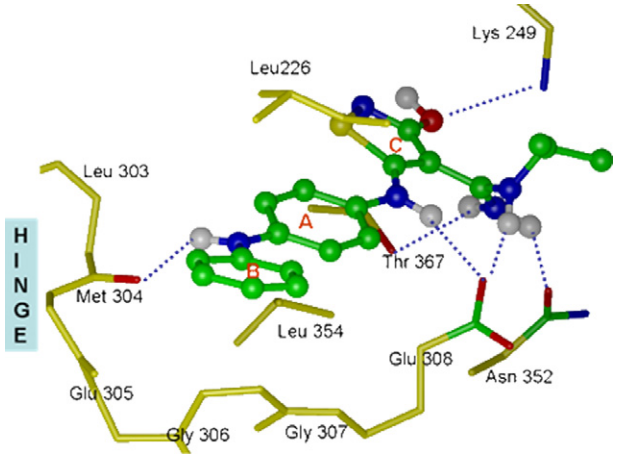


Figure 2. The predicted binding mode of **7** in the ATP site of Chk2 (PDB code: 2CN8).

The predicted pose of **7** in the ATP binding site reveals a number of key hydrogen bonds and hydrophobic interaction of Chk2 with the inhibitor. Both R^1 and R^2 are located in the vicinity of solvent-exposed area. Specifically, the NH atoms of the protonated amidine group interact extensively with side chains of Chk2 by hydrogen bonds. For example, one of its NH is engaged in the hydrogen bonding with OH of Thr367, while the other one forms a hydrogen bond with CO_2^- of Glu308 (Fig. 2). The same CO_2^- group also makes a hydrogen bond with NH located between **A** and **C** rings (Fig. 2). The NHR^1 in amidine group forms a hydrogen bond with C=O of side chain of Asn352, and the OH group on the isothiazole ring **C** hydrogen bonds with NH_3^+ of Lys249. These hydrogen bonds are similar to the contacts made by the ribose and phosphate groups of bound ADP in a typical protein kinase-ADP complex structure, and are almost identical to those hydrogen bonds between Chk2 and debromohymenialdisin inhibitor reported in the literature.[13] More importantly, the NH linker between **A** and **B** rings interacts with the backbone –C=O of Met304 by a hydrogen bond, which is the only interaction between the inhibitor and the hinge area of Chk2 (Fig. 2). Additionally, the phenyl rings in the isothiazole series of inhibitors seem to make extensive hydrophobic contacts with the side chains of Leu226 and Leu354.

These modeling results support our SAR findings. Docking results predict that both R^1 and R^2 are near solvent accessible area, suggesting the moderate effect of both R^1 and R^2 substitution on the inhibitory activity. This is in good agreement with experimental results summarized in the three tables. More importantly when the linker X = NH is altered, the sole hydrogen bond between the inhibitor and the hinge area of Chk2 is disrupted. Such a hydrogen bond interaction is believed to be crucial to achieve substantial affinity between a protein kinase and an inhibitor. This insight agrees well with our observations that compounds with various other linkers tend to be weaker or totally inactive due to the loss of NH linker at the position.

Because of the potency and selectivity our Chk2 inhibitors had achieved, we chose one representative compound **2** for further biochemical and cellular characterization. This compound was shown to suppress the ionizing radiation-induced activation of Chk2 in cells and prevented the radiation-induced Chk2-dependent degradation of HDMX protein, a negative regulator of p53. More importantly, the compound was capable of attenuating radiation induced cell apoptosis, suggesting that it does possess the radiation protective effect as we hoped. Detailed biological studies of this compound will be published elsewhere.[11]

In summary, a series of novel and selective Chk2 inhibitors were synthesized and discovered. They are simple ATP competitive inhibitors with a tractable SAR. They possess cellular activity to regulate the Chk2 mediated cell cycle arrest and apoptosis. They may be useful as a radiation protection agent in anticancer radiotherapy.

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14. Chk1 and Chk2 kinase activities were assayed using recombinant human GST-CHK1 and GST-CHK2 proteins from Upstate (Lake Placid, NY). Briefly, 10 nM of CHK1 or CHK2 was used to phosphorylate 25 μM myelin basic protein (MBP) (Invitrogen, Carlsbad, CA) in a buffer containing 8 mM MOPS, pH 7.2, 10 mM β-glycerol phosphate, 1.5 mM EGTA, 0.4 mM EDTA, 0.4 mM sodium ortho-vanadate, 100 μM ATP, 1 μCi [γ-^{33}P]ATP, 15 mM $MgCl_2$, 0.4 mM DTT, 0.006% Brij-35, 1% glycerol, and 0.2 mg/ml BSA in a final volume of 25 μl. The reaction ran for 30 min at 24 °C and was quenched by adding 100 μl of 1% trichloroacetic acid. The quenched solution was subsequently transferred to a 96-well white GF/B filter plate (Perkin-Elmer, Wellesley, MA) using a Perkin-Elmer Filtermate Universal Harvester. The radioactivity that was incorporated into MBP was trapped on the filter plate and counted using a Perkin-Elmer TopCount.