Inhibition of Chk1 by the G_2 DNA damage checkpoint inhibitor isogranulatimide

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Abstract

Inhibitors of the G_2 DNA damage checkpoint can selectively sensitize cancer cells with mutated *p53* to killing by DNA-damaging agents. Isogranulatimide is a G_2 checkpoint inhibitor containing a unique indole/maleimide/imidazole skeleton identified in a phenotypic cell-based screen; however, the mechanism of action of isogranulatimide is unknown. Using natural and synthetic isogranulatimide analogues, we show that the imide nitrogen and a basic nitrogen at position 14 or 15 in the imidazole ring are important for checkpoint inhibition. Isogranulatimide shows structural resemblance to the aglycon of UCN-01, a potent bisindolemaleimide inhibitor of protein kinase Cβ (IC_{50}, 0.001 μmol/L) and of the checkpoint kinase Chk1 (IC_{50}, 0.007 μmol/L). *In vitro* kinase assays show that isogranulatimide inhibits Chk1 (IC_{50}, 0.1 μmol/L) but not protein kinase Cβ. Of 13 additional protein kinases tested, isogranulatimide significantly inhibits only glycogen synthase kinase-3β (IC_{50}, 0.5 μmol/L). We determined the crystal structure of the Chk1 catalytic domain complexed with isogranulatimide. Like UCN-01, isogranulatimide binds in the ATP-binding pocket of Chk1 and hydrogen bonds with the backbone carbonyl oxygen of Glu^{85} and the amide nitrogen of Cys^{87}. Unlike UCN-01, the basic N15 of isogranulatimide interacts with Glu^{17}, causing a conformation change in the kinase glycine-rich loop that may contribute importantly to inhibition. The mechanism by which isogranulatimide inhibits Chk1 and its favorable kinase selectivity profile make it a promising candidate for modulating checkpoint responses in tumors for therapeutic benefit. [Mol Cancer Ther 2004;3(10):1221–7]

Introduction

Normal cells respond to DNA damage by activating cell cycle checkpoints that delay the transition from G_1 to S phase and from G_2 to M phase while DNA is repaired (1). Most cancer cells have an inoperative G_1 checkpoint due to inactivation of the *p53* tumor suppressor gene but a functioning G_2 checkpoint (2). It has been proposed that treatment with radiotherapy or DNA-damaging chemotherapeutic agents in combination with drugs that inhibit the G_2 checkpoint might promote the selective killing of tumors bearing *p53* mutations, thereby providing therapeutic benefit (3–9).

The G_2 DNA damage checkpoint comprises signal transduction cascades that link the detection of DNA breaks to inhibition of entry into mitosis via inhibition of Cdk1 and to other DNA damage responses such as the induction of DNA repair and apoptosis. Several key players in this cascade are of relevance as potential drug targets. Upstream are ATM and ATR, two large protein kinases with homology to phosphatidylinositol-3 kinases that associate with damaged DNA (10). Downstream are the protein kinases Chk1 and Chk2 (11). They are substrates of ATM and ATR and can directly phosphorylate Cdc25C, reducing its ability to carry out its major function—to activate Cdk1 by cleaving inhibitory phosphates in the ATP-binding site of Cdk1.

The first G_2 checkpoint inhibitors, caffeine and its derivative pentoxifylline, 2-aminopurine and 6-dimethylaminopurine, and staurosporine and its derivative UCN-01, were identified serendipitously (1). Subsequently, rational searches have been initiated using a cell-based assay and *in vitro* target-specific enzymatic assays. We have developed a 96-well phenotypic assay using human breast carcinoma MCF-7 cells in which *p53* is inactivated via expression of a dominant-negative *p53* mutant gene (12). The cells are arrested in G_2 by ionizing radiation and treated with chemicals or crude natural extracts. Those that contain G_2 checkpoint inhibitors force the G_2-arrested cells to enter mitosis, where they are trapped in the presence of the antimicrotubule agent nocodazole. Mitotic cells are then quantified in a modified ELISA using the mitosis-specific antibody TG-3, which recognizes a Cdk1 phosphorylation site in nucleolin (13). This assay is not target specific and has revealed several checkpoint inhibitors, including isogranulatimide and granulatimide (Fig. 1), alkaloids containing a unique indole/maleimide/imidazole skeleton (12).

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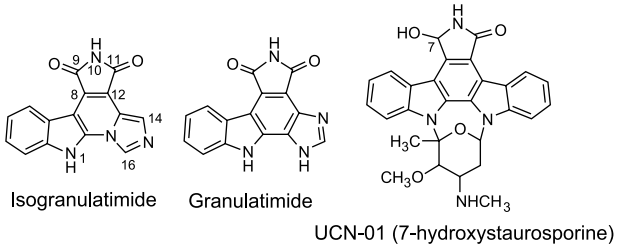

Figure 1. Structural formulas of isogranulatimide, granulatimide, and UCN-01.

UCN-01 (Fig. 1) may be considered the prototypical checkpoint inhibitor. It is the most potent of the G_2 checkpoint inhibitors identified to date (14–16), is a potent inhibitor of Chk1 (17–19) and it is being tested in clinical trials for the treatment of cancer (20). This study was undertaken to elucidate the mechanism of action of isogranulatimide and to compare its properties to those of UCN-01.

Materials and Methods

Synthesis of Isogranulatimide Analogues

Granulatimide, isogranulatimide, isogranulatimides A to C, and 17-methylgranulatimide were synthesized and purified as described (12, 21). 10-Methylisogranulatimide was obtained in moderate yield by treatment of a solution of isogranulatimide in dimethylformamide with NaH followed by iodomethane. NMR spectroscopy confirmed that alkylation occurred at N10 and not N1.

G_2 Checkpoint Assay

The ability of compounds to overcome G_2 arrest caused by ionizing radiation in MCF-7 cells expressing a dominant-negative *p53* gene was measured as described in ref. 12 using the mitosis-specific antibody TG-3 (22) and the enzyme-linked immunocytochemical assay procedure described in ref. 13. IC_{50} values for checkpoint inhibition were defined as the concentration causing half-maximal activity in this assay (12).

Protein Kinase Assays

Integrin-linked kinase 1, lymphocyte-specific kinase, mitogen-activated protein kinase kinase 1, Pim 1, p21-activated kinase 4, protein kinase Cβ, protein kinase Bα, and SRC were expressed as NH_2-terminal glutathione *S*-transferase fusion proteins in Sf9 cells using the baculovirus expression system. Extracellular signal-regulated kinase 1 and glycogen synthase kinase-3 were expressed as NH_2-terminal glutathione *S*-transferase fusion proteins in *Escherichia coli*. The proteins were purified using glutathione-agarose beads. Purified extracellular signal-regulated kinase 1 was activated *in vitro* with active mitogen-activated protein kinase kinase 1. Cdk1 was isolated from mature seastar oocytes, casein kinase II from bovine brain, DNA-dependent protein kinase from activated HeLa cells, and protein kinase A from rat muscle using conventional column chromatography. Chk1 was purchased from Upstate Biotechnology, Inc. (Waltham, MA). Chk1 assays were carried out in a 25 μL volume containing 30 ng Chk1, 2.5 μg substrate peptide (KKKVSRSGLYRSPSMPENLNRPR), 15 mmol/L β-glycerophosphate, 12 mmol/L 3-(*N*-morpholino)propanesulfonic acid (pH 7.4), 3 mmol/L EGTA, 1 mmol/L EDTA, 12 mmol/L $MgCl_2$, 150 μmol/L DTT, 3 μmol/L β-methyl aspartic acid, and 50 μmol/L γ-[^{32}P]ATP (~2,000 cpm/pmol) in the presence of compound diluted in 1% DMSO. The kinase reaction was initiated by the addition of ATP, carried out for 15 minutes at 22°C, and terminated by spotting 10 μL of the reaction volume onto a phosphocellulose Multiscreen plate (Millipore, Bedford, MA), which was washed extensively in 1% phosphoric acid. Scintillation fluid was added to the dried filter plate and radioactivity was quantitated in a Microbeta scintillation plate counter (Wallac, Gaithersburg, MD).

Other protein kinases were assayed as described in the following publications: extracellular signal-regulated kinase 1 (23), mitogen-activated protein kinase kinase 1 (24), casein kinase II (25), integrin-linked kinase 1 (26), Cdk1 (27), SRC (28), protein kinase Bα (29), protein kinase Cβ (30), Pim 1 (31), DNA-dependent protein kinase (32), lymphocyte-specific kinase (33), glycogen synthase kinase-3β (34), protein kinase A (35), and p21-activated kinase 4 (36). For IC_{50} determination, assays were carried out using a series of compound concentrations covering the IC_{50} over at least 100-fold range. The inhibition values were plotted semilogarithmically against concentration. The IC_{50} values were obtained by regression analysis using the least squares method requiring a correlation factor of at least 0.95.

Crystallization and Data Collection

The Chk1 kinase domain was expressed, purified, and crystallized as described (37). The native Chk1 kinase domain was crystallized using 9% polyethylene glycol 8000, 0.2 mol/L ammonium sulfate, and 2% glycerol at neutral pH. To soak isogranulatimide into Chk1 crystals, isogranulatimide powder was added to Chk1 crystals in crystallization solution to obtain a saturated solution and incubated for 10 days at room temperature. X-ray data were collected using the Quantum-210 detector (Area Detector Systems Corporation, Poway, CA) at the Industrial Macromolecular Crystallography Association beam line 17-ID at the Advanced Photon Source in Argonne National Laboratory (Argonne, IL). Before X-ray data collection, the crystals were equilibrated into a cryoprotectant solution containing 11% polyethylene glycol 8000 and 20% glycerol and were flash frozen in a nitrogen cold stream at −180°C. The crystal belongs to the monoclinic P21 space group with cell dimensions a = 45.0 Å, b = 65.2 Å, c = 54.2 Å, and β = 102.5° and contains one molecule per asymmetric unit. The crystal diffracted to 2.05 Å resolution. The structure was determined by difference Fourier as implemented in CNS program version 2000.1 (Molecular Simulation, Inc., San Diego, CA) using phases derived from the apo-Chk1 model (37). Fourier maps with coefficients $2|F_{obs}| - |F_{calc}|$ and $|F_{obs}| - |F_{calc}|$ were used to fit the atomic model of the inhibitor by using the computer program O version 5.9 (Uppsala University, Uppsala, Sweden). Simulated

annealing refinement followed by B-factor refinement were carried out by using the program CNX to a final R_{factor} of 0.23 and R_{free} of 0.26 at 2.05 Å resolution. The final model contains 2,224 protein atoms accounting for 276 of 289 amino acid residues of Chk1, 111 solvent atoms, and 21 inhibitor atoms.

Results and Discussion

G_2 Checkpoint Inhibition by Isogranulatimide: Importance of the Maleimide and Imidazole Rings

As a first step toward defining the mechanism of action of isogranulatimide, we determined the G_2 checkpoint inhibitory activity of synthetic analogues, concentrating on the maleimide and imidazole rings, the functional groups of which are expected to contribute most to binding specificity through hydrogen bonding.

To assess the importance of the imide nitrogen, 10-methylisogranulatimide was prepared by alkylation of isogranulatimide and tested in the G_2 checkpoint inhibition cell-based assay. It was ~15-fold less potent than isogranulatimide, showing activity only at concentrations >20 μmol/L (Fig. 2). Peak activity was observed at 200 μmol/L, compared with 10 μmol/L for isogranulatimide, followed by loss of checkpoint inhibition activity at higher concentrations, indicating that concentrations of 10-methylisogranulatimide >200 μmol/L are toxic to cells or inhibit the G_2-M transition. This result suggests that the imide nitrogen participates in hydrogen bonding with its target and/or that an increase in bulk at that position interferes with binding to the target.

Isogranulatimide shows resemblance to the aglycon portion of UCN-01 (Fig. 1). However, isogranulatimide has a maleimide group, whereas UCN-01 bears a hydroxymaleimide. We next prepared 9-hydroxyisogranulatimide (Fig. 2) as an analogue with a hydroxymaleimide. Because UCN-01 inhibits the G_2 checkpoint more potently than isogranulatimide (Table 1), we expected that the OH substitution would increase the checkpoint inhibitory activity of the analogue. However, 9-hydroxyisogranulatimide showed little checkpoint inhibitory activity at any concentration tested (Fig. 2). This may indicate a requirement for a carbonyl group at position 9 for activity. However, this compound was relatively unstable and we cannot rule out the possibility that the lack of activity could be due to compound decomposition during incubation with cells.

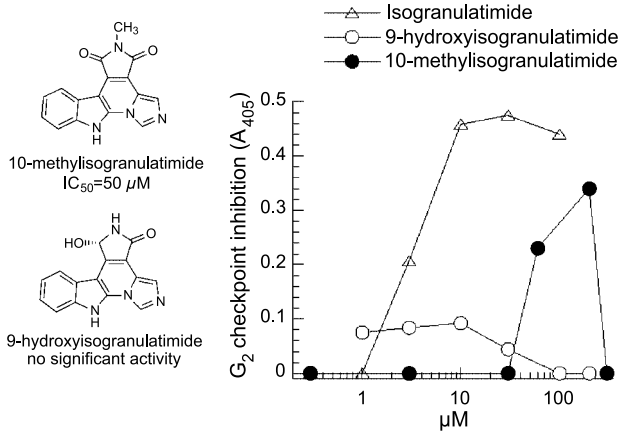

Figure 2. Checkpoint inhibitory activity of isogranulatimide, 10-methylisogranulatimide, and 9-hydroxyisogranulatimide. Sixteen hours after irradiation, G_2-arrested cells were exposed to nocodazole and to different concentrations of the compounds for 8 hours. Checkpoint inhibition was determined by enzyme-linked immunocytochemical assay as described in Materials and Methods.

Our efforts next concentrated on the imidazole ring of isogranulatimide. A second feature that distinguishes isogranulatimide from UCN-01 is a basic nitrogen in the imidazole ring, which has no counterpart in the substituted indole ring of UCN-01. The basic nitrogen's lone pair of electrons is not involved in the π bonds that contribute to the aromaticity of isogranulatimide and is thus capable of engaging in hydrogen bonds to contribute to binding affinity and specificity. In addition, the imidazole pK_a of 7.00 suggests the existence of positively charged isogranulatimide species in physiologic environments. We therefore investigated the importance of the position of the basic nitrogen in the imidazole.

The natural compound granulatimide exists as two tautomers, with a basic nitrogen at position 1 or 3, as indicated in Fig. 3. Granulatimide shows checkpoint inhibitory activity at low micromolar concentrations. We prepared isogranulatimide C, with a basic nitrogen at position 1 only and this compound also showed strong activity. We also synthesized isogranulatimide A, with a basic nitrogen at position 3 only (Fig. 3). This compound showed no significant activity, indicating that a basic nitrogen at position 1 is required. 17-Methylgranulatimide also has a basic nitrogen at position 3 only (Fig. 3) and this compound was also inactive (Fig. 3). Isogranulatimide and isogranulatimide B, with a basic nitrogen at position 2, both showed strong activity at low micromolar concentrations. This set of data indicates that a basic nitrogen at position 1 or 2 in the imidazole ring is necessary for G_2 checkpoint inhibition and that the activity of granulatimide most likely resides in the tautomer bearing a basic nitrogen at position 1. Interestingly, although the four active compounds were roughly equipotent, a drop in activity was observed at supraoptimal concentrations of granulatimide and isogranulatimide B but not isogranulatimide and isogranulatimide C, indicating that high concentrations of the latter two are not cytotoxic or do not inhibit cell cycle progression (Fig. 3).

Inhibition of Chk1 by Isogranulatimide and Analogues

The structural resemblance of isogranulatimide to the aglycon of UCN-01, a potent inhibitor of the DNA damage response kinase Chk1, suggested that isogranulatimide might also inhibit this kinase. We therefore determined the effect of isogranulatimide and the three analogues that displayed checkpoint inhibitory activity on the protein kinase activity of Chk1 *in vitro*. As shown in Table 1, isogranulatimide potently inhibited Chk1. Granulatimide

Table 1. ***In vitro*** **protein kinase inhibition profile of isogranulatimide and analogues**

	IC_{50} (μmol/L)				
	UCN-01	Isogranulatimide	Granulatimide	Isogranulatimide C	Isogranulatimide B
G_2 checkpoint	0.006	3	2	3	2
Chk1	0.007	0.1	0.25	0.65	2.3
Cdk1	0.05	10	20	17	0.5
Casein kinase II	50	50	50	>50	50
DNA-dependent protein kinase	>50	10	>50	>50	10
Extracellular signal-regulated kinase 1	>50	>50	>50	50	10
Glycogen synthase kinase-3β	0.5	0.5	2	5	0.1
Integrin-linked kinase 1	>50	>50	>50	>50	15
Lymphocyte-specific kinase	0.05	40	2	>50	2
Mitogen-activated protein kinase kinase 1	>50	>50	>50	>50	>50
Pim 1	15	50	>50	>50	50
Protein kinase A	1	>50	>50	>50	>50
p21-activated kinase 4	0.05	>50	>50	>50	>50
Protein kinase Cβ	<0.001	50	>50	>50	25
Protein kinase Bα	0.5	50	>50	>50	50
SRC	15	>50	>50	>50	50

NOTE: Data are representative of two independent experiments. Each value is the mean of triplicate assays. The error between assays was <5% for each kinase.

and isogranulatimide C were also potent Chk1 inhibitors, whereas isogranulatimide B was less potent. Given the important role of Chk1 in the G_2 checkpoint response (38), it is likely that Chk1 inhibition contributes importantly to G_2 checkpoint inhibition by isogranulatimide.

Selectivity of Isogranulatimide and Analogues toward Chk1

To evaluate the specificity of isogranulatimide and analogues, we tested their effect on the *in vitro* activity of 14 additional protein kinases and compared their inhibitory profiles with that of UCN-01 (Table 1). Isogranulatimide did not inhibit any of these kinases more potently than it inhibits Chk1; it inhibited glycogen synthase kinase-3β somewhat less potently and showed weak activity toward Cdk1 and even weaker activity toward the 12 additional kinases tested, including protein kinase Cβ. By contrast, UCN-01 was a more potent inhibitor of protein kinase Cβ than of Chk1, as reported previously (17–19, 39), and a potent submicromolar inhibitor of Cdk1, glycogen synthase kinase-3β, lymphocyte-specific kinase, p21-activated kinase 4, and protein kinase Bα.

Granulatimide and isogranulatimide B and C showed a different kinase inhibition profile than isogranulatimide (Table 1). The profile of isogranulatimide C resembled that

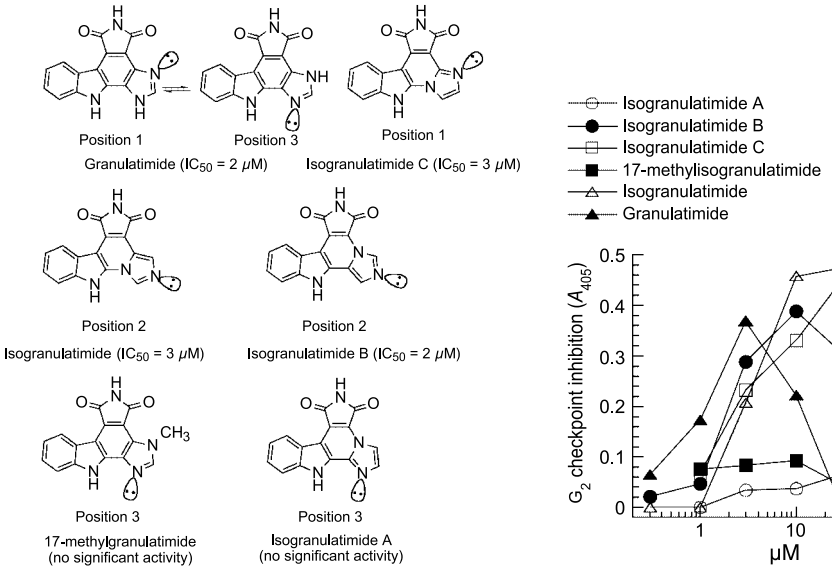

Figure 3. Checkpoint inhibitory activity of isogranulatimide analogues differing in the position of the basic nitrogen in the imidazole ring. Sixteen hours after irradiation, G_2-arrested cells were exposed to nocodazole and to different concentrations of the compounds for 8 hours. Checkpoint inhibition was determined by enzyme-linked immunocytochemical assay as described in Materials and Methods. The two tautomeric forms of granulatimide are indicated. *Double dots,* nitrogen free electrons.

of isogranulatimide, except that isogranulatimide C is a 10 times less potent inhibitor of glycogen synthase kinase-3β. The inhibitory profile of granulatimide also resembled that of isogranulatimide, except that granulatimide was a 20-fold more potent inhibitor of lymphocyte-specific kinase. Isogranulatimide B inhibited seven kinases more potently than isogranulatimide.

Overall, the specificities of isogranulatimide and UCN-01 are very different. Isogranulatimide is a less potent inhibitor of the G_2 checkpoint and of Chk1 by 2 orders of magnitude but, judging from this kinase panel, it seems to be a more selective Chk1 inhibitor than UCN-01. Notably, isogranulatimide does not inhibit protein kinase Cβ, whereas UCN-01 is a more potent inhibitor of protein kinase Cβ than Chk1. Although isogranulatimide, granulatimide, and isogranulatimide B and C are equally potent checkpoint inhibitors and all inhibit Chk1 *in vitro*, isogranulatimide and isogranulatimide C seem the most selective toward Chk1, whereas granulatimide is less selective and isogranulatimide B shows no selectivity toward Chk1. Interestingly, the broader spectrum of kinase inhibition by granulatimide and isogranulatimide B parallels their lack of checkpoint inhibition at high concentration in the cell-based assay (Fig. 3), which may reflect inhibition of additional kinases required for the G_2-M transition or for cell survival. Therefore, isogranulatimide and isogranulatimide C seem the most selective checkpoint inhibitors.

Crystal Structure of an Isogranulatimide-Chk1 Complex

To define the structural basis for inhibition of Chk1 by isogranulatimide, apo-Chk1 crystals were soaked with isogranulatimide and the structure of the complex was determined by X-ray crystallography as described in Materials and Methods. As shown in Fig. 4A, isogranulatimide binds in the ATP-binding pocket of Chk1, in one orientation only, with the imidazole facing toward the center of the ATP-binding pocket. The maleimide NH of isogranulatimide makes a hydrogen bond (3.0 Å) to the backbone carbonyl oxygen of Glu^{85}, whereas the oxygen atom at position 9 of the maleimide accepts a hydrogen bond (2.8 Å) from the amide nitrogen of Cys^{87} in the linker region. The basic nitrogen at position 15 in the imidazole ring interacts with a carbonyl oxygen in the side chain of Glu^{17}. One planar hydrophobic surface of isogranulatimide (top surface in Fig. 4A) makes many favorable van der Waals contacts with the backbone and side chains of residues from the NH_2-terminal domain of Chk1, including Leu^{15}, Val^{23}, Ala^{36}, Lys^{38}, Leu^{84}, and Tyr^{86}. The opposite hydrophobic surface (bottom surface in Fig. 4A) makes van der Waals interactions with the side chains of Val^{68}, Leu^{137}, and Asp^{148} from both NH_2- and COOH-terminal domains of Chk1.

For comparison, the crystal structure of the Chk1-UCN-01 complex (37) is shown in Fig. 4B. The two inhibitors form similar hydrogen bonds with the backbone carbonyl oxygen of Glu^{85} and the amide nitrogen of Cys^{87}. Unlike isogranulatimide, UCN-01 also interacts with Ser^{147} through its hydroxyl group and with Glu^{91} and Glu^{134} through its tetrahydropyran ring. In the Chk1-UCN-01

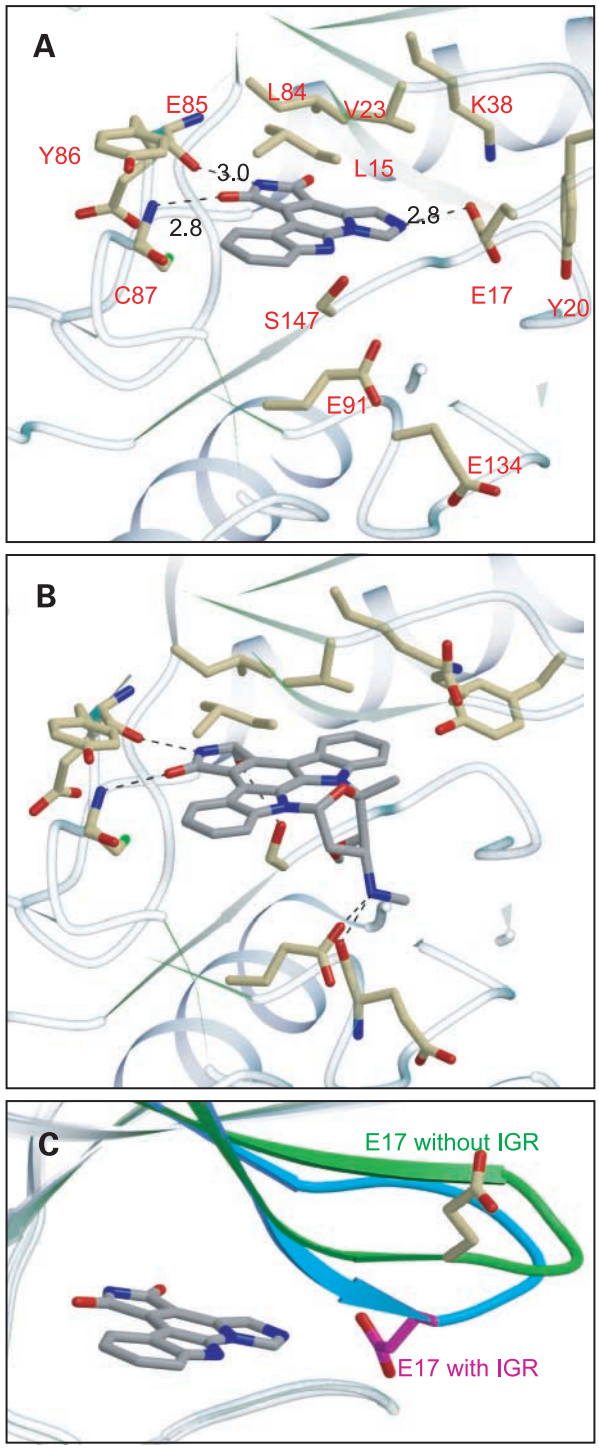

Figure 4. Structural basis for Chk1 inhibition by isogranulatimide. **A,** binding of isogranulatimide in the active site of Chk1: Chk1 atoms involved in binding to isogranulatimide or to UCN-01 binding. *Dashed lines,* hydrogen bonds between isogranulatimide and Chk1 (in Å); *gray,* inhibitor carbons; *beige,* enzyme carbons; *red,* oxygens; *blue,* nitrogens. **B,** binding of UCN-01 in the active site of Chk1. **C,** conformation change in the Chk1 glycine-rich loop induced by isogranulatimide binding. *Green,* glycine-rich loop in the absence of isogranulatimide (*IGR*); *blue,* glycine-rich loop in the presence of isogranulatimide. Only the side chain of Glu^{17} is displayed.

complex, the side chain of Tyr^{20} bends inward toward the inhibitor molecule, whereas in the Chk1-isogranulatimide complex the side chain of Tyr^{20} turns away from the active site (see Fig. 4A and B).

Interestingly, the isogranulatimide-Chk1 complex differs significantly from the structures of the native Chk1 and of the UCN-01-Chk1 complex with respect to Glu^{17}, which is in the enzyme's glycine-rich loop. In native Chk1 and in the Chk1-UCN-01 complex, the side chain of Glu^{17} points away from the enzyme active site, whereas in the isogranulatimide-Chk1 complex the Glu^{17} side chain points toward isogranulatimide and interacts with the basic N15 of the inhibitor. Glu^{17} is flanked on both sides by glycine residues, making this segment of the protein flexible. The conformational change in the glycine-rich loop caused by isogranulatimide binding is illustrated in Fig. 4C. The Chk1-isogranulatimide and Chk1-UCN-01 complexes also differ from each other in the side chain conformations of residues Glu^{91}, Phe^{93}, Glu^{134}, Ser^{147}, and Asp^{148}. In addition, compared with the Chk1-UCN-01 binary complex, isogranulatimide is shifted slightly (by ~ 0.5 Å) outward from the pocket to avoid close contact between the maleimide carbonyl oxygen and the side chain of Leu^{84}.

This crystal structure helps interpret the isogranulatimide structure-activity profile observed in our cell-based G_2 checkpoint inhibition assay. The maleimide NH and the maleimide C9 carbonyl are necessary for activity and they are both involved in hydrogen bonding with Chk1. The interaction between the basic N15 of isogranulatimide and Glu^{17} explains why isogranulatimide binds Chk1 with a single mode—this interaction would not occur if the indole were in the position of the imidazole, and binding affinity would be considerably reduced. The crystal structure may also explain the role of the basic N15 in isogranulatimide in checkpoint inhibition. Because imidazole has a pK_a of 7.00, it is possible that N15 bears a positive charge in the complex that adds to the strength of the hydrogen bond with the negatively charged carboxylate of Glu^{17}. Given the general importance of the glycine-rich loop in the binding of ATP and substrate to kinases (40), isogranulatimide may inhibit Chk1 both by directly competing with ATP and by hindering interaction with substrate proteins. The structure may also reveal why 9-hydroxyisogranulatimide is not more active than isogranulatimide as a checkpoint inhibitor: the unique orientation of isogranulatimide in the ATP-binding site, with the imidazole facing toward the center of the ATP-binding pocket, puts the hydroxyl group at position 9 on the side of the maleimide ring where it cannot interact with Ser^{147}.

This study defines the structural elements of isogranulatimide required for activity as a G_2 checkpoint inhibitor and a Chk1 kinase inhibitor. Current evidence indicates that Chk1 activity is required for the G_2 checkpoint, whereas Chk2 plays a smaller supportive role (38). It is likely that the most significant checkpoint target of isogranulatimide is Chk1 because it is a 40-fold less potent inhibitor of Chk2 than Chk1 and it does not inhibit ATM or ATR,[7] although the involvement of other unidentified targets cannot be excluded. The results also show that isogranulatimide is a less potent but more selective kinase inhibitor than UCN-01, making it a promising candidate for modulating checkpoint responses in tumors for therapeutic benefit. This study also shows that a suitably designed phenotypic assay can be a powerful drug discovery tool to identify chemicals with pharmacologically desirable properties, such as cell permeability, stability in cellular environments, and suitable selectivity profile, which are difficult to design or to engineer into traditional *in vitro* target-specific enzymatic assays.

[7] Unpublished data.

Acknowledgments

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