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Radioprotection by Hymenialdisine-Derived Checkpoint Kinase 2 Inhibitors

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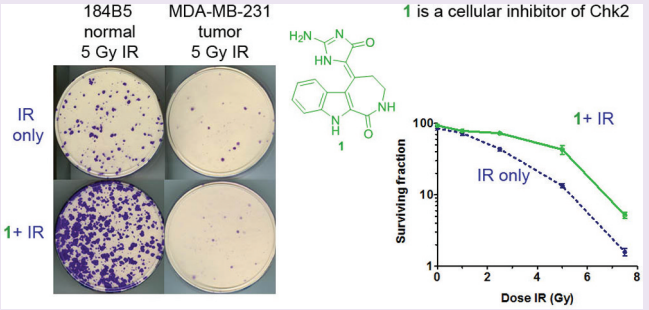
Supporting Information

ABSTRACT: DNA damage induced by ionizing radiation activates the ataxia telangiectasia mutated pathway, resulting in apoptosis or DNA repair. The serine/threonine checkpoint kinase (Chk2) is an important transducer of this DNA damage signaling pathway and mediates the ultimate fate of the cell. Chk2 is an advantageous target for the development of adjuvant drugs for cancer therapy, because inhibition of Chk2 allows normal cells to enter cell cycle arrest and DNA repair, whereas many tumors bypass cell cycle checkpoints. Chk2 inhibitors may thus have a radioprotective effect on normal cells. We report herein a class of natural product derived Chk2 inhibitors, exemplified by indoloazepine **1**, that elicit a strong ATM-dependent Chk2-mediated radioprotection effect in normal cells and p53 wt cells, but not p53 mutant cells (>50% of all cancers). This study represents the first example of a radioprotective effect in human cells other than T-cells and implicates a functional ATM pathway as a requirement for IR-induced radioprotection by this class of Chk2 inhibitors. Several of the hymenialdisine-derived analogues inhibit Chk2 at nanomolar concentrations, inhibit autophosphorylation of Chk2 at Ser516 in cells, and increase the survival of normal cells following ionizing radiation.



Cells are often exposed to various forms of DNA damage from sources including reactive oxygen species, ultraviolet light (UV), background radiation, and environmental mutagens. DNA damage from these external sources can lead to an accumulation of oncogenic mutations and genomic instability. Cells carry out DNA damage responses in order to repair the genetic damage through checkpoints that can delay cell cycle progression or modulate DNA replication.[1,2] The processes involved in DNA repair are therefore crucial in maintaining the integrity of the genome and preventing tumorigenesis.[3,4]

The DNA damage response pathways are necessary for the coordination of checkpoint and DNA repair processes as well as apoptosis or cell senescence.[3−5] Following DNA damage, the DNA sensors ataxia telangiectasia mutated (ATM) and ataxia-telangiectasia (ATR) are activated by DNA damage.[6] However, ATR and ATM respond to different forms of DNA damage. ATR is activated by tracts of single strand breaks in conjunction with its partner protein ATR-interacting protein (ATRIP).[7−9] ATM is recruited and activated by double strand breaks (DSBs) in association with the MRE11:Rad50:NBS1 (MRN) sensor complex.[9,10] Because ATM and ATR have a sequence homology, they often have overlapping functions and substrate specificities.[11]

ATR is activated most strongly when DNA replication is interrupted, such as nucleotide depletion or replication-blocking DNA damage lesions often induced by UV light.[12,13] Replication-blocking causes DNA polymerases to become uncoupled from the replicative helicase,[14] which generates single strand (ss) breaks and quickly gets coated with a trimeric ssDNA-binding protein complex, Replication Protein A (RPA). ATR is then recruited and activated at the single strand breaks with its partner ATRIP.[9,13] The activation of ATR does not require autophosphorylation or posttranslational modification.[12]

The ATM pathway is activated by ionizing radiation (IR) and genotoxins that induce DSBs. They can also be activated weakly, if at all, by agents that block DNA replication without inducing damage.[15] When DNA damage is not present, ATM is believed to exist as inactive homodimers. When DSBs occur, the inactive ATM homodimers are quickly induced to autophosphorylate *in trans*, causing them to dissociate to form partially active monomers.[16] The first autophosphorylation site identified was Ser1981. This residue is not necessary for ATM function;[17] however, its modification is linked to the activation of ATM.[16] The monomers of ATM are recruited to DSBs *via* interactions with the MRN sensor complex,[18] which fully activates ATM, allowing it to act on downstream substrates.

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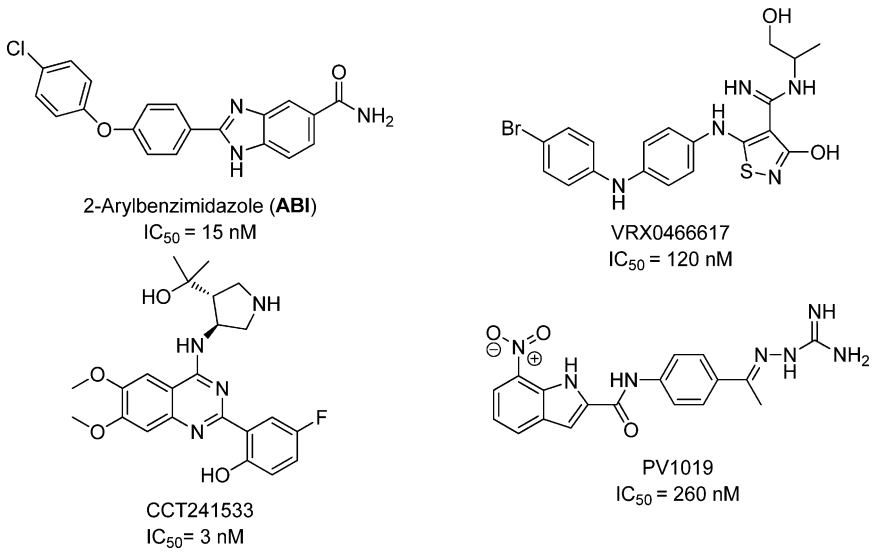

Figure 1. Current inhibitors of Chk2.

The kinase substrates of the DNA damage sensors, ATR and ATM, include checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2), respectively. Both of these checkpoint kinases tightly regulate the cell cycle checkpoints.[19,20] Temporary arrest of the cell cycle allows for repair and prevention of the replication of damaged DNA or, alternatively, the induction of apoptosis or terminal cell cycle arrest in the event of heavily damaged cells.[21−24] Once ATR is activated, it phosphorylates its downstream effector Chk1 at both Ser345 and Ser317, in response to UV DNA damage.[25,26] Phosphorylation at Ser345 is essential for Chk1 biological activity, although the exact modifications that are required for regulation of the Chk1 catalytic activity are poorly understood.[13,27] Chk1 can also be phosphorylated by ATM in response to IR-induced replication stress, contributing to the degradation of Cdc25A following IR.[4] Thus, Chk1 and Chk2 possess some overlapping functions despite their lack of structural similarity.[28−30]

Following ATM activation, ATM phosphorylates several downstream effectors responsible for checkpoint controls, including Chk2.[4,31] This major DNA damage response mediator is a serine/threonine kinase that plays a key role in modulating the DNA damage signal following IR-induced DSBs.[1,4,32,33] The function of Chk2 has been proposed to act as a barrier to tumorigenesis by preventing or delaying genetic instability following the early activation of the DNA damage response in cancer lesions.[34,35] Chk2 is directly activated and phosphorylated by ATM at Thr68,[15,36] causing homodimerization and activation through *trans*-autophosphorylation[37,38] on Thr383 and Thr387[39,40] and subsequently *cis*-autophosphorylation on Ser516.[39] Activation of ATM-Chk2 by IR leads to phosphorylation of several downstream substrates involved in many cellular processes including cell cycle arrest, apoptosis, and DNA repair.[21,41−46]

Chk2 has become an attractive target for cancer therapy given its pivotal role in the regulation of the cell cycle, apoptosis, and DNA repair following DNA damage.[32,47] Ionizing radiation and DNA-damaging chemotherapeutics continue to be the conventional form of cancer therapy, often resulting in severe side effects. Inhibiting the DNA damage checkpoints could potentially augment the current chemo- or radiation therapies by sensitizing cancer cells to the cytotoxic effects of the therapy. Conversely, checkpoint inhibition could potentially allow an enhancement in survival of normal cells by desensitizing normal cells from IR or DNA-damaging agents.[48]

Chk2 inhibition is involved in eliciting radio- or chemoprotection of normal tissue *via* the inhibition of p53-dependent apoptosis.[32,47] Most tumor cells bypass normal cell cycle responses following DNA damage if p53 is mutated.[49] In normal cells, p53 is activated by Chk2 following IR, resulting in cell cycle arrest and apoptosis.[43,44,50] Thus, inhibition of Chk2 in normal cells may prevent p53-mediated apoptosis, therefore promoting DNA repair and potential cell survival. This hypothesis was supported by a study in which Chk2 −/− transgenic mice showed a reduction in p53-dependent apoptotic responses.[51] Increased survival of the Chk2 −/− mice was observed following exposure to whole body IR as compared to Chk2 +/+ mice.[51] Inhibition of Chk2 in mouse thymocytes[52] and isolated CD4+ and CD8+ human lymphocytes[53] also showed a decrease in apoptosis in response to IR. These and other elegant studies[31,52−56] therefore indicate that inhibition of Chk2 in normal cells may increase survival following IR by selectively reducing p53-mediated cell death.

There are only a few small molecule inhibitors of Chk2 that have been reported in the literature. Of these reported inhibitors, high-throughput screening has proven to be very useful resulting in the identification of several ATP-competitive Chk2 inhibitors including 2-arylbenzimidazole-5-carboxamides (**ABI**),[53,54] isothiazole carboxamidines,[31,52] and bis-guanylhydrazones (Figure 1).[55,57] Interestingly, many of these Chk2 inhibitors display exciting but varying results in terms of radioprotection of normal cells and sensitization of cancerous cells with varying p53 deficiencies. For example, Chk2 inhibition has been shown to enhance the effects of cytotoxic drugs.[58−60] Transfection of MCF-7 cells with Chk2 siRNA[61] was found to enhance the effect of paclitaxel.[60] Chk2 inhibition augmented the levels of mitotic catastrophe when used together with doxorubicin[62] or cisplatin[63] by releasing mitochondrial pro-apoptotic proteins. In addition, the small molecule Chk2 inhibitor PV1019 (Figure 1) also showed potentiation of DNA-damaging agents topotecan and camptothecin in OVCAR-4 and OVCAR-5 human tumor cells.[57]

The use of Chk2 inhibitors for potentiating DNA-damage agents continues to be exciting albeit somewhat controver-

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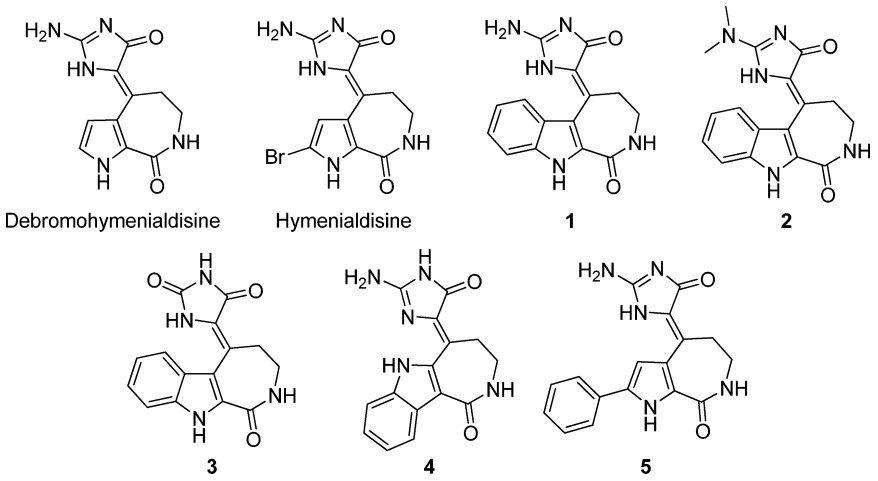
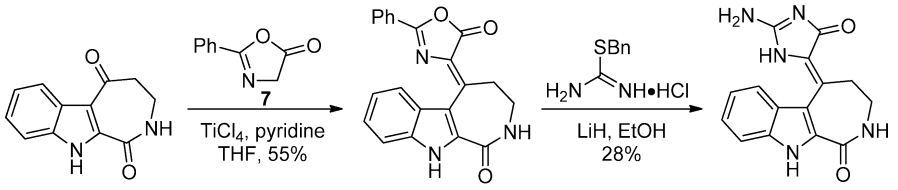
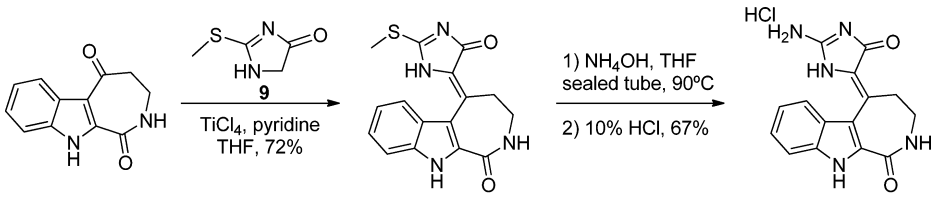


Figure 2. Structures of debromohymenialdisine, hymenialdisine, and hymenialdisine derived analogues **1**−**5**.

Scheme 1. Previously Reported Synthesis of Indoloazepine 1[66,67]



Scheme 2. Improved Synthesis of Indoloazepine 1



sial[5,32,47] as not all inhibitors of Chk2 demonstrate these effects.[31,52] For example, the potent Chk2 inhibitor CCT241533 (Figure 1) does not potentiate the cytotoxicity of a selection of genotoxic agents but potentiates the selectivity of PARP inhibitors in p53-defective cancer cell lines.[64,65] Recent studies also demonstrate that inhibition of Chk2 without an additional genotoxic agent may also be advantageous for therapeutic development in tumors possessing increased levels of activated Chk2.[32,57,65] Tumor cells in which Chk2 is constitutively activated have plausibly adapted a Chk2 dependence in order to survive. Studies by Pommier *et al.* demonstrated the antiproliferative activity of Chk2 inhibitor PV1019 (Figure 1) as well as Chk2 siRNA, in cancer cell lines such as KM12, OVAR-3, and OVAR-4 that overexpressed Chk2.[57] Thus, interest in discovering new Chk2 inhibitors as tools or therapeutic leads, in order to unravel the different cellular responses, remains high.

We report herein a class of natural product derived Chk2 inhibitors, exemplified by indoloazepine **1**, that elicit a strong ATM-dependent Chk2-mediated radioprotection effect in normal cells and p53 wt cells, but not p53 mutant cells (>50% of all cancers). These studies represent the first example of a radioprotective effect in human cells other than T-cells, and unlike many of the reported checkpoint kinase inhibitors, indoloazepine **1** does not induce significant cell cytotoxicity in the NCI 60 cell line screen, including KM12, OVAR-3, and OVAR-4 cells (Supporting Information, Table S1). These compounds present therefore significant potential as tools to study ATR/ATM-mediated radioprotection pathways or as potential pharmaceutically relevant lead structures.

We have previously reported the synthesis of hymenialdisine-derived indoloazepine Chk2 inhibitor **1**.[66] We report herein an improved synthetic route that allows access to multiple derivatives (**1**−**5**, Figure 2) in an efficient manner and their respective *in vitro* and cellular kinase assay activity. Indoloazepine **1** was further evaluated for its cellular mechanism and its efficacy as a radioprotecting agent in cancerous and normal breast cells, in response to ionizing radiation.

RESULTS AND DISCUSSION

Syntheses of Hymenialdisine-Derived Analogues.

Our group previously reported the synthesis of **1** (Scheme 1), which utilized a titanium(IV)-mediated aldol condensation of indoloaldisine **6** with phenylazlactone **7** in 55% yield.[66,67] Compound **8** was unstable and difficult to purify, contributing to its modest yields. Compound **8** was subsequently treated with *S*-benzylthiourea under basic conditions giving **1** (15%

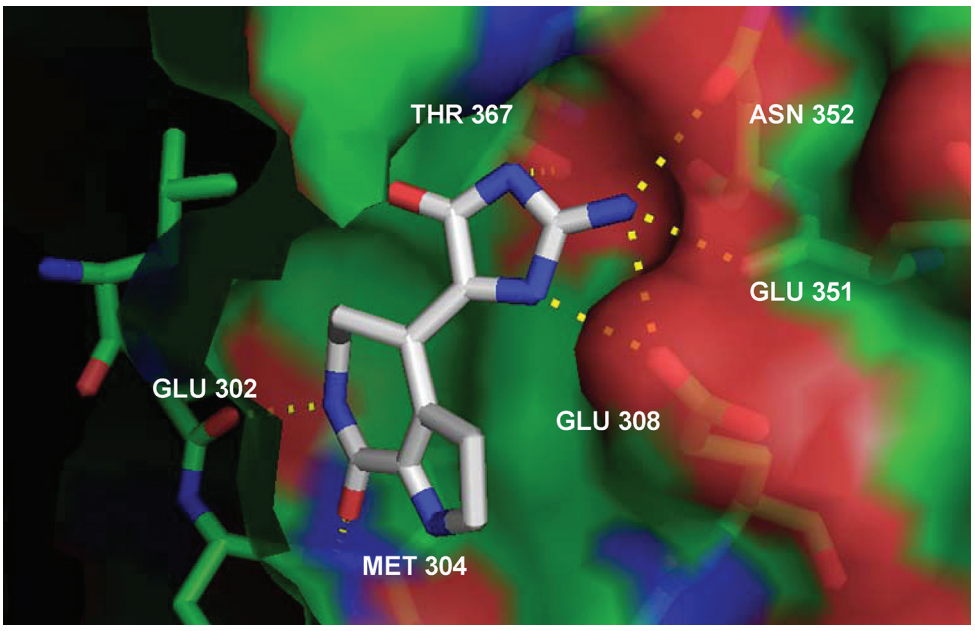
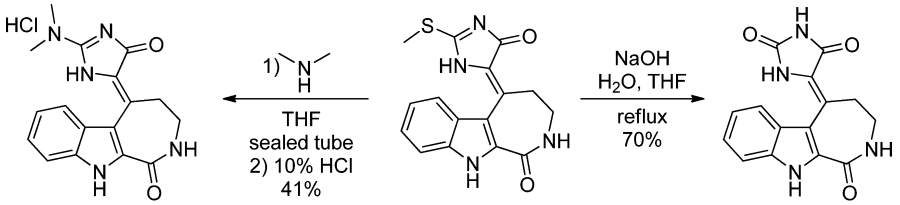


Figure 3. Crystal structure of debromohymenialdisine in the Chk2 catalytic domain (PDB code: 2CN8). The enzyme residues are depicted in green stick form, whereas the inhibitor is depicted in gray stick form. Nitrogen atoms are depicted in blue and oxygen in red. The yellow dashes represent hydrogen bonds. Green surface areas represent uncharged residues; positive residues are depicted in blue and negative in red.[38]

Scheme 3. Synthesis of Analogues 2 and 3



yield over two steps) and required several chromatographic purifications following the final step.[66,67]

An improved synthetic route involved the use of *S*-methyl-2-thiohydantoin **9**.[68−70] This new route has many advantages over previously reported approaches to these types of hymenialdisine-type natural products, including increased yields, ease of purifications, and stable intermediates, as well as a versatile intermediate **10**, from which many analogues can be readily derived. Thiohydantoin **9** (Supporting Information, Scheme 1) was coupled to indoloaldisine **6**[66,67] in a titanium(IV) chloride mediated condensation to yield the stable analogue **10** in 72% yield (Scheme 2). Ammonium hydroxide was stirred with **10** in a sealed tube, and the resulting precipitate was collected as the HCl salt, affording **1** in 67% yield (48% yield over the last two steps) and not requiring further purification by column chromatography.

Analogues **2** and **3** were synthesized to assist in understanding the importance of the free amine moiety of the glycocyamidine ring in the ATP binding pocket. Crystallographic data of the binding interactions between indoloazepine **1** and Chk2 are not reported; however, on the basis of the observed biological activity from the analogues we present herein, we postulate that the binding is similar to the earlier reported co-crystal structure of debromohymenialdisine (Figure 2) and Chk2 (Figure 3).[38] This co-crystal structure indicates key hydrogen bonding interactions between the exocyclic amine group of the glycocyamidine ring and the carbonyl oxygens of ASN352. Analogue **2** possesses a dimethyl amine that removes the possibility of hydrogen bonding interactions with ASN352 and GLU351 and adds steric bulk, both of which could negatively impact interactions in the binding pocket. Analogue **3** contains a hydantoin ring instead of a glycocyamidine ring, abolishing its ability to act as a hydrogen bond donor. However, the additional oxygen could interact within the binding pocket as a hydrogen bond acceptor (Figure 3).[38]

Our new route was ideal to render efficient and practical syntheses of these analogues by reacting different nucleophiles with the versatile intermediate **10** (Scheme 3). Reacting **10** with dimethylamine in a sealed tube followed by an acid wash afforded **2** (Scheme 3, 41% yield). Compound **3** was synthesized by refluxing **10** with sodium hydroxide in water/THF (Scheme 3, 70% yield).

Previously, replacement of the pyrrole moiety of hymenialdisine with an indole ring (indoloazepine **1**) improved the potency and kinase selectivity as a Chk2 inhibitor (Figure 2).[66] On the basis of this result, there was interest in directing our synthetic efforts toward new analogues **4** and **5** (Figure 2). Analogue **4** (*iso*-indoloazepine) is the constitutional analogue of **1**, which contains an inverted indole ring in which the nitrogen is directed toward the glycocyamidine ring, possibly providing additional hydrogen bonding opportunities with GLU308

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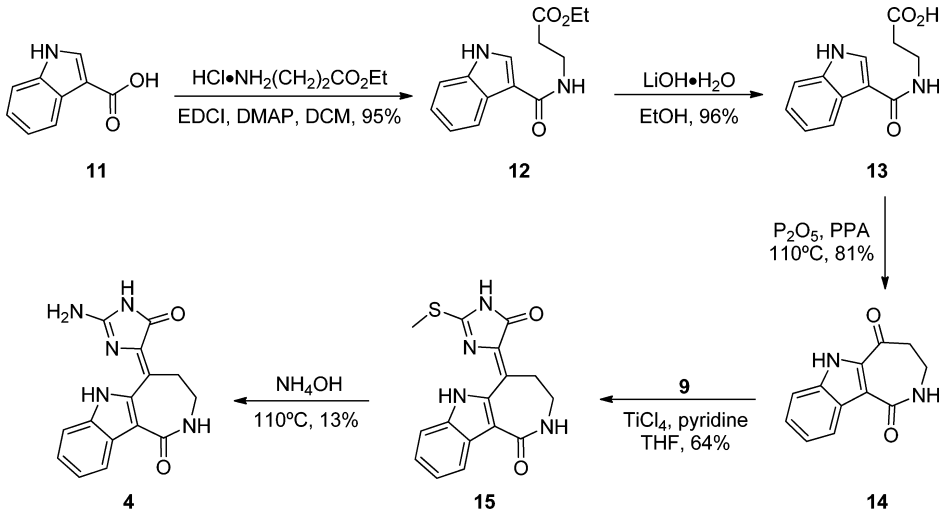
Scheme 4. Synthesis of *iso*-Indoloazepine 4



(Figure 3). Analogue **5** contains a phenyl group that replaces the bromine of hymenialdisine and was designed in an attempt to occupy the hydrophobic area within the ATP binding pocket (Figure 3).

Scheme 4 illustrates the synthesis of **4** beginning with an EDCI coupling of indole-3-carboxylic acid **11** to β-alanine ethyl ester to obtain **12**. Ester **12** was hydrolyzed with lithium hydroxide followed by a Friedel–Crafts ring closure with phosphorus pentoxide and polyphosphoric acid to give the seven-membered ring **14**. *S*-Methyl-2-thiohydantoin **9** was coupled to **14** using titanium(IV) chloride to afford **15**. The glycocyamidine ring was completed by heating **15** with ammonium hydroxide in a sealed tube to yield analogue **4**. Analogue **4** was successfully crystallized in methanol (Figure 4).

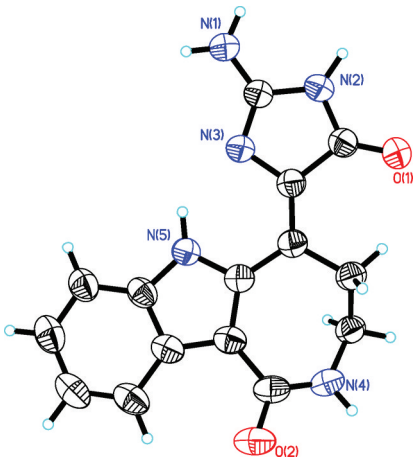


Figure 4. X-ray crystal structure of *iso*-indoloazepine **4**.

The yield associated with the final step is arguably quite low (13%), which is likely due to the decrease in conjugation of the double bond with the carbonyl in the glycocyamidine ring. This is postulated to be the result of an intramolecular hydrogen bonding interaction of the N-3 nitrogen with the indole proton (2.66 Å), resulting in a less electrophilic C-2 carbon and a preference of the double bond between the C-2 and N-3 positions (Figure 4).

The synthesis of **5** utilized ethyl ester **16**, which was synthesized as previously reported.[71] A Suzuki coupling was performed under basic conditions, which also hydrolyzed the ester in the same reaction to yield **17** (Scheme 5).[72] The seven-membered ring **18** was achieved as described above, followed by the titanium(IV) chloride mediated coupling with **9** to afford **19** (73% yield). The final product **5** was obtained by heating **19** in a sealed tube with ammonium hydroxide in THF.

Evaluation of Hymenialdisine-Derived Analogues for Inhibition of Purified Chk1 and Chk2. Analogues **1**–**5** were evaluated for their ability to inhibit the related checkpoint kinases Chk1 and Chk2 using a radioactive-based filter assay *in vitro* with purified Chk1 and Chk2 enzymes (Supporting Information, Table S4 and Figure S1). Figure 5 illustrates the dose-dependent inhibition of Chk2 (Figure 5a) and Chk1 (Figure 5b) by **1**. The IC_{50} values of analogue **1** are 13.5 nM (Chk2) and 220.4 nM (Chk1), respectively (Table 1). Neither analogue **2** nor **3** indicated any inhibition of Chk1 or Chk2 at relevant concentrations (IC_{50} >10 μM). However, compounds **4** and **5** were also found to be potent inhibitors of Chk2, albeit with somewhat less selectivity for Chk2 over Chk1. The results of the Chk1 and Chk2 inhibition by compounds **1**–**5** are summarized in Table 1.

The lack of Chk2 inhibition by compound **2** suggests that the methyl groups add steric bulk to the exocyclic nitrogen of the glycocyamidine ring, which could prevent a proper fit in the active pocket of Chk2 and eliminate the hydrogen bonding interactions with ASN352, as seen in Figure 3.[38] Replacement of the exocyclic amine with an oxygen also abrogated all activity. The lack of activity of compounds **2** and **3** indicates that the free amine group is necessary for binding and could also act as a hydrogen bond donor, consistent with the binding of hymenialdisine to Chk2. Compounds **1**, **4**, and **5** all possess the free amine on the glycocyamidine ring allowing for the hydrogen bonding that is not present in **2** and **3**. The decrease in potency of compound **4** relative to **1** may potentially be explained by its crystal structure (Figure 4). The crystal structure of **4** clearly illustrates that the double bond of the glycocyamidine ring is not conjugated with the carbonyl in the ring but instead is conjugated to the indoloaldisine portion, thus competing for hydrogen bonding with GLU308. The indole hydrogen could form an intramolecular hydrogen bond with the proximal nitrogen, potentially reducing hydrogen bond opportunities to the Chk2 catalytic site. As anticipated, phenyl pyrrole **5** was capable of inhibiting Chk2 effectively but

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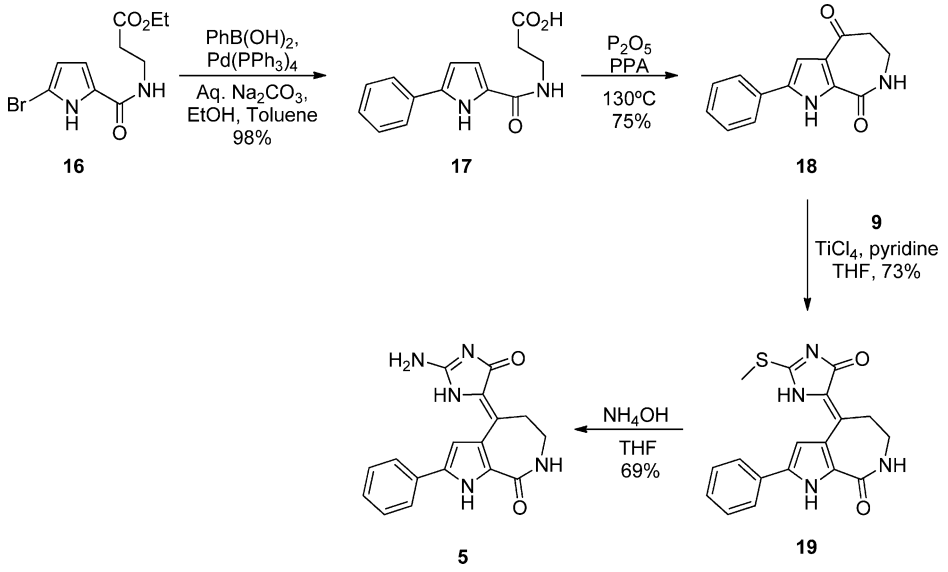
Scheme 5. Synthesis of Hymenialdisine Derivative 5



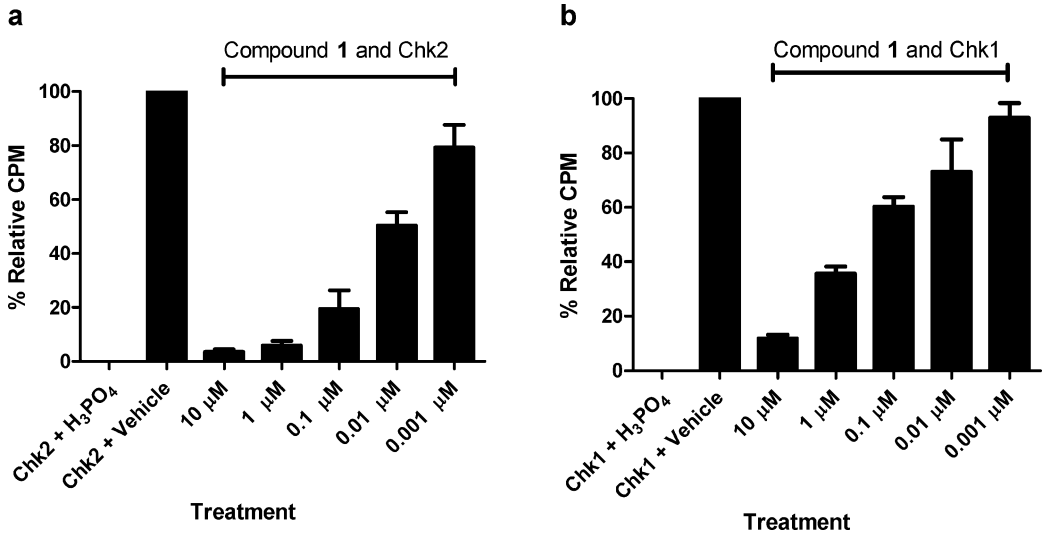


Figure 5. Inhibition of [γ-^{33}P]-ATP phosphorylation by purified active Chk1 or Chk2. H_3PO_4 was used as a negative or untreated control. (a) Inhibition of [γ-^{33}P]-ATP phosphorylation of Chk2 by indoloazepine **1**. (b) Inhibition of [γ-^{33}P]-ATP phosphorylation of Chk1 by indoloazepine **1**. The data are expressed as mean ± SEM from 3 independent experiments.

Table 1. Summary of IC_{50} Values for Kinase Inhibition by Analogues 1−5

kinase	1	2	3	4	5
Chk1 (nM)	220.4	>10,000	>10,000	581.4	626.8
Chk2 (nM)	13.5	>10,000	>10,000	65.9	53.9

unfortunately displayed a reduced activity and selectivity compared to indoloazepine **1**. Thus, combined, these studies indicate that for future scaffold optimization the glycocyamidine ring plays a key role in H-bonding interactions and minor changes can abrogate activity completely; however, the pyrrolic portion of the hymenialdisine scaffold appears to less involved in binding and may provide a handle for further derivatization to obtain higher substrate specificity.

In order to determine the selectivity of **1** for Chk2, we expanded our previous kinase screen, and additional *in vitro* kinase-profiling experiments were performed (Supporting Information, Table S2). The kinases listed were selected from a large panel of kinases known to be affected by the hymenialdisines at low micromolar concentrations.[73,74] The inhibitory activity of **1** over a panel of cellular kinases indicates that **1** exhibits good selectivity toward Chk2. Only 3 other kinases showed some inhibition by **1** in nanomolar concentrations, but their IC_{50} values were approximately 10-fold greater than those for Chk2. These data demonstrate the specificity of **1** for Chk2.

Evaluation of Cellular Inhibition of Chk2. In order to determine support for cellular inhibition of Chk2 by **1**, we evaluated Chk2 (total protein levels), Chk2 Thr68 and Chk2 autophosphorylation at Ser516. A normal breast cell line, 184B5 was left untreated or pretreated for 2 h prior to IR with vehicle (DMSO), the positive control Chk2 inhibitor **ABI** (Figure 1, 30 μM), the negative control Chk2-inactive analogue **2** (30 μM), and the Chk2 inhibitor **1** (30 μM). The cells were irradiated (10 Gy) and treated with fresh media and 30 μM compound (vehicle, **ABI**, **2**, or **1**) for 4 h at which time whole

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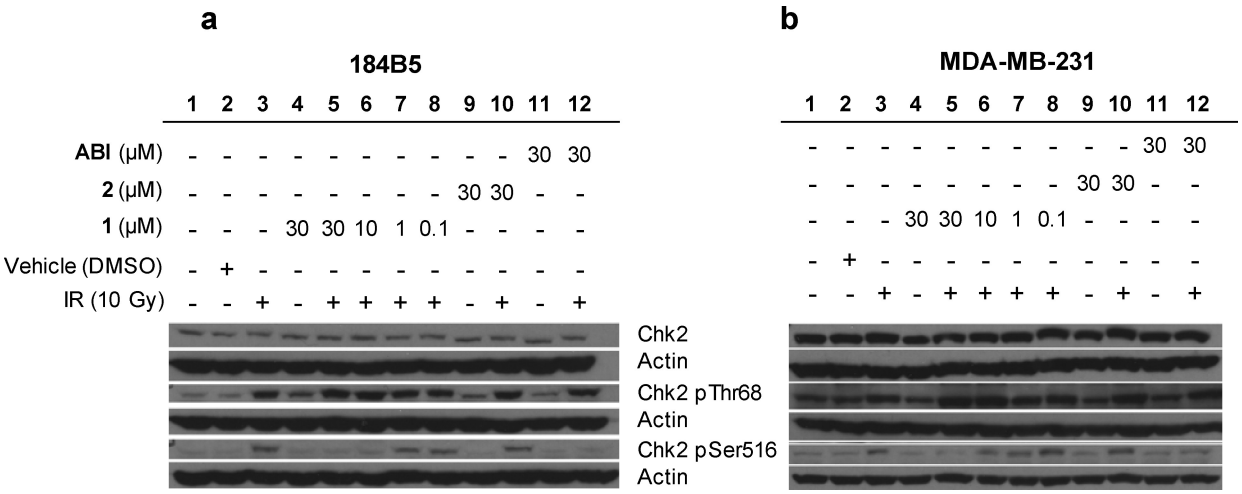

Figure 6. Western blot analysis of 184B5 (Figure 6, panel a, normal, p53 WT) and MDA-MB-231 (Figure 6, panel b, tumor, p53 mutant) whole cell extracts untreated (control) or treated with vehicle or with compounds **ABI**, **2** and **1** for 2 h prior to IR (10 Gy). Cells were incubated for 4 h after IR when extracts were collected. The Western blot was evaluated with Chk2 antibodies. These blots are representative of 3 independent experiments.

cell extracts were collected. Whole cell extracts were evaluated by Western blot for cellular inhibition of Chk2. Total Chk2 levels were probed with the corresponding Chk2 antibody and did not show inhibition in the level of Chk2 with low (0.1 μM) and high (30 μM) concentrations of **1** (Figure 6a). **ABI** (30 μM) also did not inhibit total levels of Chk2. It should be noted that, unlike most Chk2 inhibitors, even at the high dose (30 μM), compound **1** did not exhibit any cell cytotoxicity in this cell line (data not shown). The IR-induced activation of Chk2 was confirmed by observing activation of Chk2 at Thr68, which occurred when cells were exposed to DNA-damaging IR (Figure 6a, Lanes 3, 5–8, 10, and 12). Unlike the Chk2 inhibitor VRX0466617,[75] inhibition of Chk2 by **1** or **ABI** did not affect phosphorylation of Thr68 by ATM (Figure 6a, Lanes 5 and 12), indicating that the ATM function was not affected by compound **1.** However, there was a dose-dependent effect as a result of compound **1** on IR-induced Chk2 autophosphorylation at Ser516 shown in 184B5 cells (Figure 6a, lanes 5–8). As expected, Chk2 autophosphorylation at Ser516 was also seen with **ABI** (Figure 6a, lane 12), a known Chk2 inhibitor.[53] **ABI** also showed a level of Chk2 inhibition at 30 μM comparable to that seen with **1**. There was no inhibition of Chk2 phosphorylation seen for either Thr68 or Ser516 with the inactive Chk2 agent **2** (IC_{50} > 10 μM), which was used in the assay as a negative control (Figure 6a, Lane 10).

To further investigate the effect of **1** on Chk2 inhibition, we evaluated **1** in a breast tumor cell line with a mutated p53. DNA damage was induced by IR in MDA-MB-231 cells (breast tumor, p53 mutant), and inhibition of Chk2 phosphorylation was evaluated (Figure 6b). As in the normal breast cell line, levels of total Chk2 remain unchanged when treated with IR, **ABI**, **2**, or **1**. Chk2 Thr68 was phosphorylated following treatment with IR and remained unchanged when pretreated with **ABI**, **2**, or **1** (Figure 6b, Lanes 3, 5–8, 10, 12). Chk2 inhibition with **1** inhibited autophosphorylation at Ser516 in a dose-dependent manner following IR (Figure 6b, Lanes 5–8). Levels of Chk2 inhibition were again compared to **ABI**, which also inhibited *cis*-autophosphorylation of Chk2 at Ser516 (Figure 6b, Lane 12). As anticipated, inactive analogue **2** did not inhibit Chk2 autophosphorylation (Figure 6b, Lane 10). These data illustrate that the cellular inhibition of Chk2 by **1** is independent of the p53 status of the cell lines and that Chk2 is inhibited in both the normal breast cell line, 184B5, as well as the breast tumor cell line, MDA-MB-231.

Analogues **4** (IC_{50} = 65.9 nM) and **5** (IC_{50} = 53.9 nM) also demonstrated inhibition of Chk2 in the radioactive-based filter assay (Table 1). To evaluate whether these analogues were capable of cellular inhibition of Chk2, we evaluated autophosphorylation of Chk2 at Ser516 (Figure 7). Both

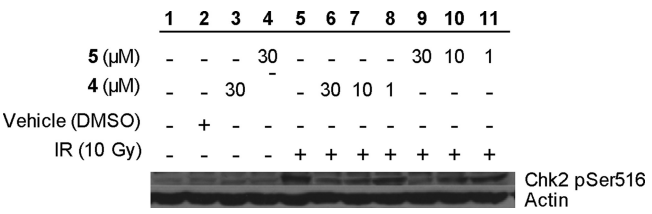

Figure 7. Western blot analysis of 184B5 whole cell untreated (control) or treated with vehicle or with compounds **5** and **4** for 2 h prior to IR (10 Gy). Cells were incubated for 4 h after IR when extracts were collected. The Western blot was evaluated with Chk2 antibodies. These blots are representative of 3 independent experiments.

analogues **4** and **5** demonstrated a dose-dependent effect on inhibition of Chk2 Ser516, supporting cellular inhibition of Chk2 by **4** and **5**.

These data indicate that the *in vitro* activity of inhibitors **1**, **4**, and **5** translates into cell culture. In addition, we show that the more potent and selective Chk2 inhibitor **1** abrogates Chk2 activity in both the normal breast cell line as well as the breast tumor cell line.

Evaluation of Indoloazepine as a Protecting Agent against IR. Chk2 inhibition has been indicated to elicit radio- or chemoprotection of normal tissue *via* inhibition of p53-dependent apoptosis.[32,47] Therefore, we performed a clonogenic survival assay to determine if pretreatment of normal breast cells (184B5 cells, p53 wt) with Chk2 inhibitor **1** (30 μM) was capable of protecting only the normal cells from the cytotoxic effects of various doses of IR (0–7.5 Gy). We hypothesized that the inhibition of Chk2 would have an insignificant effect on cell survival in p53 mutant cancer cells (MDA-MB-231 cells), compared to the vehicle control.

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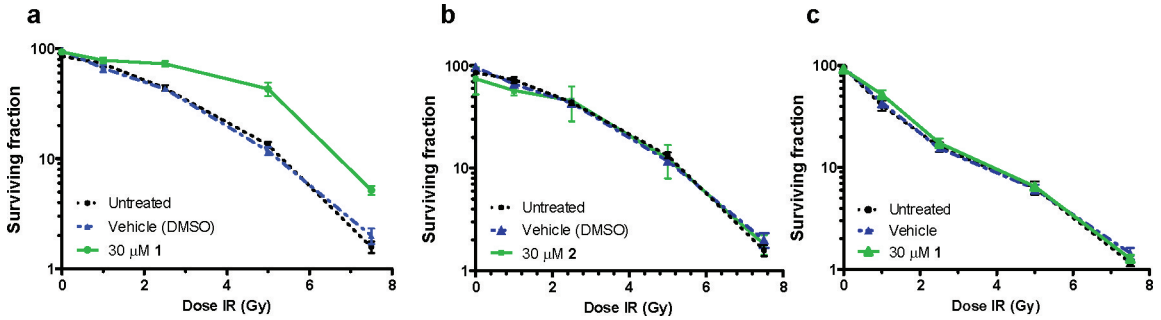

Figure 8. Cloning survival of cells untreated or pretreated for 30 min with 30 μM **1** or **2** followed by IR. (a) 184B5 cells were treated with **1** and IR (0−7.5 Gy); (b) 184B5 cells were treated with **2** and IR; and (c) MDA-MB-231 cells were treated with **1** and IR. The data are expressed as mean ± SEM from 3 independent experiments.

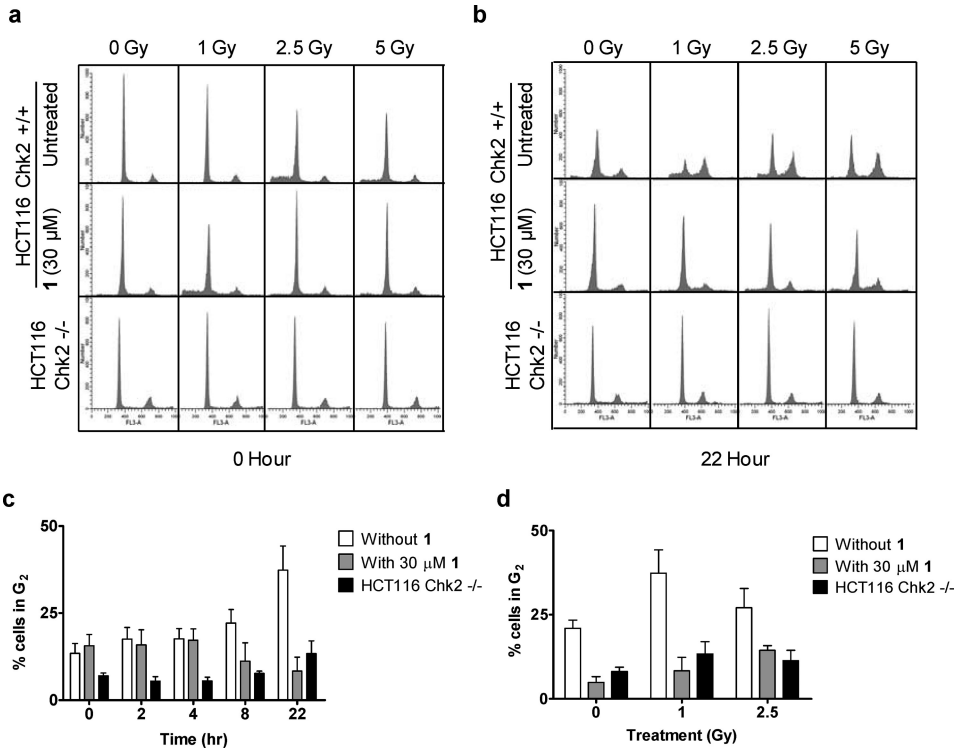

Figure 9. DNA frequency histograms of HCT116 Chk2 +/+ or Chk2 −/− cells either untreated (control) or pretreated for 2 h with **1** (30 μM) followed by IR (0−5 Gy). (a) Cells harvested and fixed at 0 h after IR. (b) Cells harvested and fixed 22 h after IR. DNA histograms were obtained following cell staining with propidium iodide. The cell cycle distributions (G1, S, and G2) were analyzed using the ModFit program. (c) Data for G_2 following 1 Gy IR. (d) Data for G_2 at 22 h following various dose levels of IR. Data for cell cycle histograms are expressed as mean ± SEM from 3 independent experiments.

In order to evaluate this hypothesis, 184B5 and MDA-MB-231 cells were left untreated or pretreated with vehicle (DSMO) or **1** (30 μM) for 30 min. The cells were irradiated in suspension (0−7.5 Gy) and seeded at cloning density. Cells were allowed to incubate for 7 days at which point fresh media was given to the clones. After 10−14 days, clones were fixed and stained with crystal violet. Surviving clones were scored (*i.e.*, colonies containing >50 cells), and viability of the cells was determined (Figure 8).

The results of the clonogenic assay revealed that the control treatment (vehicle only) resulted in a decrease in the number of viable cells with increasing doses of IR (Figure 8a) as anticipated. However, treatment with Chk2 inhibitor **1** dramatically enhanced survival in the normal cell line (p53 wt), as compared to the vehicle control treatments with nearly 75% survival at doses up to 5 Gy IR, compared to the untreated control. It should be noted that the cells were only exposed to compound **1** for 30 min. The negative control, inactive Chk2 inhibitor analogue **2**, did not exhibit any radioprotective effects in the IR-challenged 184B5 cells (Figure 8b). These data strongly indicate that the enhanced survival seen in this cell line is due to the abrogation of Chk2 activity by Chk2 inhibitor **1**.

Importantly, pretreatment of the p53 mutant MDA-MB-231 cells for 30 min with or without **1** (30 μM) followed by the same doses of IR (0−7.5 Gy) did not show an enhancement in survival in the presence of Chk2 inhibitor **1** (Figure 8c). Figure 8c clearly indicates that there was no detectable induction of cell survival as the untreated, vehicle, and treated cells showed statistically identical survival curves. The results in these two cell lines implicate the involvement of the p53 pathway in the survival mechanism of Chk2 inhibitor **1**, in cells treated with IR.

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The clonogenic survival assays in the normal and tumor cell lines demonstrated that inhibition of Chk2 in p53 mutant breast cancer cells did not have any cellular effect on cell survival, whereas Chk2 inhibition by **1** increased cell survival following IR-induced DNA damage in the normal breast cell line. The graphs in Figure 8 clearly indicate the difference in IR protection by **1** in the p53 wt cells over the p53 mutant cells with only 30 min of exposure to compound **1**. To further implicate the role of p53 in this survival pathway, we evaluated p53wt MCF-7 breast cancer cells in the same clonogenic survival assay and found that compound **1** induce a similar radioprotective effect in this p53 wt cell line (Supporting Information, Figure S2).

Evaluation of the Requirement of the ATM Pathway for Checkpoint Kinase Dependent Radioprotection. In order to understand the importance of the ATM pathway in the checkpoint kinase mediated radioprotection observed by compound **1**, we repeated the clonogenic survival assay in HCT116 cells. HCT116 cells were chosen for this study because of their known defects in the ATM signaling pathway and their reliance on the ATR pathway for survival following DNA damage.[76−78] Our results demonstrate that indoloazepine **1** did not affect IR-induced survival of ATM-deficient HCT116 cells (Supporting Information, Figure S3) at varying doses of radiation. Consistent with this, Chk2 −/− HCT116 cells also did not induce survival compared to the wild type control at varying doses of radiation (Supporting Information, Figure S3). These data illustrates that an active ATM pathway is required for the radioprotective properties elicited by checkpoint kinase inhibitor, indoloazepine **1**, in response to ionizing radiation. In addition, this data suggests that any ability of indoloazepine **1** to affect the ATR- Chk1 pathway did not elicit a significant effect related to either cell survival in this IR-treated cell line.

Evaluation of Indoloazepine in the Cell Cycle. Checkpoint signaling pathways are activated following DNA double strand breaks and result in the arrest of cells at the G_1/S, S, or G_2/M transitions, which allows cells time to repair the damage or execute apoptosis.[79] In response to IR, the activated ATM-Chk2 pathway phosphorylates their downstream substrates resulting in a G_2 arrest. To determine whether inhibition of Chk2 by **1** would modulate the G_2 checkpoint, we monitored the cell cycle using single parameter flow cytometry. Although both cancerous cell lines, the availability of HCT116 cell lines with both Chk2 +/+ and Chk2 −/− phenotypes allowed us to further verify Chk2 as the target of **1**. We hypothesized that inhibiting Chk2 with **1** would yield similar cell cycle effects as the Chk2 −/− cells following IR-induced DNA damage.

HCT116 Chk2 +/+ cells were left untreated or treated with **1** (30 μM) for 2 h, at which point cells were irradiated with various doses of IR. HCT116 Chk2 −/− remained untreated and were exposed to the same doses of IR as the HCT116 Chk2 +/+ cells. The cells were allowed to incubate for 0−22 h after which time they were harvested and fixed. Consistent with the early and temporary activation of the ATM-Chk2 pathway,[44,45,80] untreated HCT116 Chk2 +/+ cells resulted in an induction of G_2 arrest 22 h after IR, relative to the control cells (Figure 9a,b; see also Supporting Information, Figures S4 and S5). However, HCT116 Chk2 +/+ cells displayed a significant ($^*p < 0.0145$) decrease in the G_2 checkpoint when the cells were treated with Chk2 inhibitor **1** (Figure 9c) and was apparent at various doses of IR (Figure 9d). A similar abrogation of the G_2 checkpoint was demonstrated in the Chk2 −/− cell line (Figure 9c and d). The similarities with respect to the inhibition of the IR-induced G_2 checkpoint arrest in the Chk2 −/− cell line are consistent with our data that **1** inhibits Chk2 in the wild type cells. Abrogation of the IR-induced G_2 arrest with Chk2 inhibitor **1** allows normal cells to progress to the G_1 checkpoint where DNA replication is initiated and carried out. Inhibition of Chk2 by compound **1** did not have a statistically significant effect on IR-induced change in the G_1 compared to the no-IR treated controls (see Supporting Information, Figures S4 and S5). However, considering the ability of indoloazepine **1** to also inhibit Chk1 (see Figure 5 and Supporting Information, Figure S6), we do not wish to eliminate the possibility of indoloazepine **1** mediating IR-induced G2 arrest, *via* modulation of Chk1-mediated cell cycle signaling.

Conclusion. Following ionizing radiation, the DNA sensors ATR and ATM are activated and phosphorylate checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2), respectively. Both of these checkpoint kinases tightly regulate the cell cycle checkpoints and ultimately the cellular response to the DNA damage. A few classes of Chk2 inhibitors have been reported in the literature, with varying phenotypic responses in terms of cell cytotoxicity, radioprotective effects, and synergism when combined with mechanistically different chemotherapeutics.

We describe herein an efficient synthesis of the hymenialdisine-derived indoloazepine **1** and several of its analogues **2**−**5**. These analogues have provided a model for binding in the Chk2 binding pocket and demonstrated the importance of the glycocyamidine moiety and the key role of the free amine for inhibition of Chk2. The newly synthesized analogues **4** and **5** inhibit Chk2 at nanomolar levels in a radioactive filter-based kinase assay. Inhibition of purified Chk2 by compounds **1**, **4**, and **5** translated well into the cellular inhibited *cis*-autophosphorylation at Ser516 of Chk2, thus demonstrating a direct cellular inhibition of Chk2. Chk2 inhibitor **1** also demonstrated inhibition of IR-induced G_2 arrest in HCT116 Chk2 +/+ cells, consistent with the decrease of IR-induced G_2 arrest in Chk2 −/− cells. Inhibition of Chk2 did not have an effect on IR-induced arrest of G_1. Unlike other potent Chk2 inhibitors (Figure 1), compound **1** does not induce significant levels of cell cytotoxicity, including cell lines expressing high levels of Chk2 (KM12, OVAR-3, and OVAR-4). More importantly, 30-min exposure of cells to indoloazepine **1** resulted in a dramatic increase in survival in normal cells exposed to IR, whereas tumor cells having mutated p53 or ATM-deficient pathways had no noticeable enhancements in survival. These studies indicate that these agents require a functional ATM-dependent Chk2 pathway in order to elicit a radioprotective effect in p53 wild type cells. These data therefore warrant further investigations into the possible therapeutic potential of Chk2 inhibitors with respect to the radioprotection of normal cells in response to IR. In summary, indoloazepine **1** elicits a potent radioprotective effect in normal cells but not p53 mutant cancerous cells, *via* an ATM-Chk2-dependent signaling pathway following activation by IR.

METHODS

General Methods. The 184B5 and MDA-MB-231 cell lines (ATCC, Manassas, VA) were cultured in DMEM supplemented with 5% heat-inactivated bovine calf serum and supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 0.2 mM L-glutamine, and 100 mM MEM nonessential amino acids. HCT116 cells were cultured in McCoy's 5A with 5% bovine calf serum

and supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin. The HCT116 cell lines were a generous gift from Bert Vogelstein (Howard Hughes Medical Institute, Chevy Chase, MD). For all lysate and whole cell experiments, the inhibitors were dissolved in DMSO, and the final DMSO concentration of the experiments was maintained at ≤0.1% in the reaction buffer or culture medium. Cells were cultured at 37 °C in a 5% CO_2 incubator. Cells in the cloning survival assay and Western blots were irradiated with a ^{60}Co source providing 1.93 Gy/min. Cells used in the cell cycle analysis were irradiated using a 6 MV linear accelerator (Clinac 2100 C, Varian Inc.) at a dose rate of 300 cGy/min. The quantity of incorporated [γ-^{33}P]-ATP on the P30 filter mat was detected using a Wallac 1450 Microbeta Plus Liquid Scintillation counter. Cell cycle analysis was performed using a Vantage TurboSort SE cytometer (Becton Dickinson, San Jose, CA).

Preparation and Characterization of Checkpoint Kinase Analogues. Analogues **1**−**5** were synthesized as described in the Supporting Information and stored as solids at −20 °C.

***In Vitro* Kinase Assay.** In a final reaction volume of 25 μL, Chk1 (h) or Chk2 (h) (Millipore) (5−10 mU) was incubated with 8 mM MOPS pH 7.0, 0.2 mM EDTA, 100 μM CHKtide (KKKVSRSGLYRSPSMPENLNRPR) (Millipore), [γ-33P-ATP] (Perkin-Elmer) (approximately 1 μCi diluted in 25 mM Mg acetate and 0.25 mM ATP) and inhibitor. The reaction was initiated by the addition of the MgATP mix. After incubation for 10 min at 30 °C, the reaction was stopped by addition of 5 μL of a 3% phosphoric acid solution. Ten microliters of the reaction was spotted onto a P30 filtermat and washed three times for 5 min in 75 mM phosphoric acid and once in methanol for 2 min before the addition of scintillation cocktail. The filtermat was read in a scintillation counter.

Kinase Profile Assay. The Kinase profile was performed by the Millipore Kinase Profiling Services (Millipore, Billerica, MA). This technology is a radioisotope-based P30 filter-binding assay. **1** was dissolved in pure DMSO to make a 10 mM stock. **1** was then diluted in pure DMSO to make serial dilutions based on the IC_{50} ranges with a final DMSO concentration of less than 1%. The purified enzyme (*e.g.*, Chk2) was diluted with 20 mM MOPS/NaOH pH7.0, 1 mM EDTA, 0.01% Triton X-100, 5% glycerol, 0.1% 2-mercaptoethanol, 1 mg/mL BSA and incubated with 8 mM MOPS pH 7.0, 0.2 mM EDTA, substrate [*e.g.*, Chktide (100 μM in H_20) (KKKVSRSGLYRSPSMPENLNRPR)] and 10 mM MgAcetate and [γ-^{33}P-ATP] (specific activity approximately 500 cpm/pmol, concentration as required). The reaction was initiated by the addition of the MgATP mix. After incubation for 40 min at RT, the reaction was stopped by the addition of 3% phosphoric acid solution. Ten microliters of the reaction was then spotted onto a P30 filtermat and washed three times for 5 min in 75 mM phosphoric acid and once in methanol prior to drying and scintillation counting.

Western Blot Analysis of Whole Cell Extracts of Cells Treated with Chk2 Inhibitor. 184B5 cells in T25 flasks were 80% confluent when incubated with **1** (in DMSO, 0.1%) for 2 h and were then activated *in situ* with 10 Gy of IR. Cells were given fresh media and inhibitor and incubated for 4 h. Media was then removed from the plates, and cells were washed two times with ice-cold PBS. Whole cell lysis buffer (20 mM Tris HCl (pH = 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 20 μM DTT, 2 μM $NaVO_4$, 2 μM NaF, 2 μM PMSF, 2 μM β-glycerophosphate, 0.02 μg/mL Aprotinin, 0.02 μg/mL Leupeptin) was then added to the plates, which were then rocked for 10 min at 4 °C. Cells were then scraped and transferred to a tube followed by brief sonication. Cells were centrifuged at 500 × g for 3 min. 50 μg of protein (quantified with Bradford assay) was diluted with 6X SDS-PAGE buffer. Equal amounts of denatured protein were electrophoresed on SDS-polyacrylamide gels and transferred to PVDF membranes using wet transfer. Membranes were blocked with 4% blocking grade nonfat dairy milk (NFDM) for 1 h at RT and washed with Tris-buffered saline-Tween 20 (TBS-T) containing 10X Tris buffer and 0.1% Tween 20 for 10 min. Blots were then probed with primary AB 1:1000 in 5% BSA TBS-T (overnight, 4 °C) and were washed with TBS-T (3 × 5 min). They were then incubated with horse radish peroxidase conjugated to secondary antibody. Actin antibody (1:10,000 dilution) (Amersham) was incubated in 4% NFDM in TBS-T, washed, and incubated with horse radish peroxidase conjugated to secondary antibody mouse. Complexes were detected by enhanced chemiluminescence (ECL) technique according to manufacturer's directions (Amersham) on XAR film.

Cloning Survival Assay. 184B5 cells were cultured, and when 80% confluent, cells were treated with **1** for 2 h and irradiated in suspension with varying doses (0−7.5 Gy) of irradiation (Cobalt60) on ice. Cells were diluted, plated at cloning density in triplicate, and allowed to incubate at 37 °C with 5% CO_2 and ambient oxygen. Medium was replaced at 7 days, and clones were incubated another 3−7 days at which point clones were fixed with 0.9% saline and stained with 3% crystal violet in 70% ethanol. Surviving clones were scored (*i.e.*, colonies containing >50 cells), and viability of the cells was determined.

Flow Cytometry. The distribution of HCT116 cells at different stages of the cell cycle was estimated using flow cytometric DNA analyses. Cells (5×10^5) were incubated overnight in McCoy 5A medium containing 5% BCS and then treated with or without compound for 2 h in 6-well plates. Tissue culture plates were placed on a 1.5 cm sheet of tissue equivalent bolus material, to provide for dose buildup on the radiation treatment couch. The linac gantry was positioned at 180° to irradiate the plates using a single portal from below. Dose was calculated to the bottom of the tissue culture plates. Cells were irradiated with 1.0, 2.5, or 5.0 Gy at a dose rate of 3.0 Gy/min. Cell were allowed to incubate for varying lengths of time (0, 2, 4, 8, 22 h) postirradiation. Cells were then harvested and washed with phosphate-buffered saline (PBS; pH 7.4) containing 5% BCS, fixed with 70% ethanol/30% water at 4 °C. The cells were washed with 5% BCS/PBS twice, and the pellet was resuspended in a solution containing RNase (20 μg/mL) and propidium iodide (Invitrogen) and incubated for 15 min in the dark at 37 °C. For each sample, at least 10,000 events were analyzed using a Vantage TurboSort SE cytometer (Becton Dickinson, San Jose, CA) with 488 nM excitation, fluorescence at 630/22 bandpass filter. The percentage of cells in each cell cycle phase was calculated using CellQuest Ver3.3, and the WinList6 and ModFITLT software packages (Becton Dickinson).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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