Inhibition of the G₂ DNA Damage Checkpoint and of Protein Kinases Chk1 and Chk2 by the Marine Sponge Alkaloid Debromohymenialdisine*

Cells can respond to DNA damage by activating checkpoints that delay cell cycle progression and allow time for DNA repair. Chemical inhibitors of the G₂ phase DNA damage checkpoint may be used as tools to understand better how the checkpoint is regulated and may be used to sensitize cancer cells to DNA-damaging therapies. However, few inhibitors are known. We used a cell-based assay to screen natural extracts for G₂ checkpoint inhibitors and identified debromohymenialdisine (DBH) from a marine sponge. DBH is distinct structurally from previously known G₂ checkpoint inhibitors. It inhibited the G₂ checkpoint with an IC₅₀ of 8 μM and showed moderate cytotoxicity (IC₅₀ = 25 μM) toward MCF-7 cells. DBH inhibited the checkpoint kinases Chk1 (IC₅₀ = 3 μM) and Chk2 (IC₅₀ = 3.5 μM) but not ataxiatelangietasia mutated (ATM), ATM-activated related protein, or DNA-dependent protein kinase in vitro, indicating that it blocks two major branches of the checkpoint pathway downstream of ATM. It did not cause the activation or inhibition of different signal transduction proteins, as determined by mobility shift analysis in Western blots, suggesting that it inhibits a narrow range of protein kinases in vivo.

DNA damage activates signal transduction pathways called checkpoints, which delay cell cycle progression and allow more time to repair DNA (1–3). Checkpoints arrest cells in the G₁ phase to prevent replication of damaged DNA and in the G₂ phase to prevent the segregation of damaged chromosomes during mitosis.

The G₂/M transition is controlled by the Cdc2 protein kinase. During G₂ arrest, Cdc2 is inactivated through phosphorylation of Thr-14 and Tyr-15 in its ATP-binding site by protein kinases including Wee1 and Myt1 (4, 5). Entry into mitosis requires dephosphorylation of these sites by Cdc25 phosphatases. According to our current understanding of the G₂ checkpoint, DNA damage activates the ATM,¹ and ATR members of the phosphoinositide kinase family (6, 7). A signal then is transmitted through the downstream protein kinases Chk1 and Chk2 (6–11), which are able to phosphorylate Cdc25 on Ser-216. This phosphorylation is thought to directly prevent Cdc25 from activating Cdc2 kinase (12) or to separate Cdc25 from Cdc2 kinase by promoting the association of Cdc25 with 14–3–3 proteins (10, 13–16). Chk1 and Chk2 also can phosphorylate and activate Wee1, a kinase that catalyzes Cdc2 inhibitory phosphorylation (17, 18). Chk1 is required for initiating G₂ arrest (19, 20). Chk2 can phosphorylate p53 on Ser-20 in vitro (21–23), and p53 targets such as p21 and 14–3–3σ have roles in maintaining G₂ arrest (24, 25). These results as well as experiments with knockout mice (21) are consistent with a role of Chk2 in maintaining G₂ arrest.

Our understanding of checkpoints stems mainly from genetic studies in yeast and mice, in vitro studies with amphibian egg extracts, and studies of human syndromes associated with predisposition to cancer. Compounds that inhibit the G₂ checkpoint may be useful additional tools to study the checkpoint mechanism in mammalian systems. G₂ checkpoint inhibitors also may be valuable in cancer therapy to enhance the effectiveness of DNA-damaging agents in tumors with a defective G₁ DNA damage checkpoint, such as those with mutated p53 (26–29). However, few G₂ checkpoint inhibitors are known. Those found so far include caffeine and 1-substituted caffeine analogs (30–35), 2-aminopurine, and 6-dimethylaminopurine (36), staurosporine, 7-hydroxyxstatosporine, SB-218078 (37–39), and isogranulatimide (29). All have been shown to enhance the cytotoxicity of DNA-damaging agents (29, 39). Staurosporine is a broad specificity protein kinase inhibitor, and 7-hydroxyxstatosporine is an in vitro inhibitor of several protein kinases (40–42) including Chk1 (43, 44). 7-Hydroxyxstatosporine is being evaluated in phase I clinical trials for the treatment of cancer (45). Caffeine and caffeine analogs have many pharmacological activities (35) including in vitro inhibition of ATM and ATR protein kinase activity (3, 12, 46–48), but they are not considered drug candidates.

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†† To whom correspondence should be addressed: 2146 Health Sciences Mall, Vancouver, British Columbia, Canada V6T 1Z3. Tel.: 604-822-2304; Fax: 604-822-5227; E-mail: michel@otter.biochem.ubc.ca.

¹ The abbreviations used are: ATM, ataxiatelangietasia mutated protein; DBH, debromohymenialdisine; HPLC, high pressure liquid chromatography; mp53, mutant p53; MTI, S(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PK, protein kinase; DNA-PK, DNA-dependent protein kinase; SAPK, stress-activated protein kinase; ATR, ATM-Rad3-related protein.
Experimental Procedures

Extraction and Isolation of DBH and Related Compounds—Specimens of S. flabeliformis were collected in the waters off Motupore Island in Papua New Guinea. The samples were frozen on site and transported to Vancouver over dry ice. A frozen sample (87 g wet weight) was thawed and extracted exhaustively with methanol. The methanol extract was filtered and concentrated in vacuo to give a dark brown solid. This crude extract was fractionated on a Sephadex LH-20 column using methanol as the eluent. Active fractions were identified using the G2 checkpoint inhibition assay (29) and subjected further to silica gel-flash chromatography using stepwise gradient elution (CH3Cl to 1:1 CH3Cl/methanol saturated with NH3). Further purification was achieved by repeated fractionation on reversed-phase HPLC using 80:20:0.5 water/methanol/trifluoroacetic acid as the eluent. The pure fractions of DBH and hynemialdisine were converted from trifluoroacetic salts to the hydrochloride salts by the addition of 3 N HCl followed by concentration under reduced pressure. Debromopyrrolococlam was obtained by subjecting DBH to a 5% K2CO3 solution followed by concentration in vacuo. It was then purified using HPLC with 80:20 water/methanol as the eluent. The related compounds 2-aminoimidazole and 2-amino-4,5-imidazole-dicarboxonitrile were obtained from Aldrich.

G2 Checkpoint Inhibition Assay—The assay was performed as described in Ref. 29 using human mammary tumor MCF-7 cells expressing a dominant negative mutant p53 (mp53) gene. The number of cells that escaped G2 arrest and became trapped in mitosis was determined by examining linked immunosorbent assay using the TG-3 antibody (29, 49) or by counting mitotic cells using fluorescence microscopy (50).

MTT Cell Proliferation Assay—MCF-7 mp53 cells were seeded at 1000 cells/well in 96-well plates, grown overnight, and treated or not treated with DBH for 24 h. The drug was removed, and cells were allowed to grow in fresh medium until those not treated with the drug approached confluence, which was typically 4–6 days. Cell proliferation was measured as follows: 25 μl of a 5 mg/ml solution of 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in phosphate-buffered saline was added to cells in the presence of 100 μl of cell culture medium. After a 2-h incubation at 37 °C, 100 μl of 20% sodium dodecyl sulfate dissolved in dimethylformamide/water (1:1), pH 1.1, was added, and the absorbance at 570 nm was measured after overnight incubation.

Cell and Nuclear Extracts and Western Blotting—Cells were harvested by trypsinization, and cell pellets were washed once with phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride. Cells were suspended in 20 volumes of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml each of leupeptin, pepstatin, and aprotinin, 30 μg/ml DNase I and RNase A, 1 mM dithiothreitol, and 1 mM sodium orthovanadate) and lysed by pipetting up and down 20 times, incubating on ice for 15 min, pipetting up and down again 20 times, and leaving on ice for another 15 min. Lysates were cleared by centrifugation at 15,000 × g for 15 min at 4 °C. Nuclei were prepared as described in Ref. 51 and lysed in SDS-sample buffer. Fifty μg of protein was resolved by 10% SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose by electroblotting, and blocked in 5% Carnation dry milk in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5% Tween 20 for 1 h at room temperature. The nitrocellulose membrane was probed with Cdc2 rabbit antiserum (KAP-CC001, StressGen Biotechnologies, Victoria, Canada) in blocking solution overnight at 4 °C. The membrane was washed three times and incubated with horseradish peroxidase-conjugated goat antibodies to rabbit IgG (13858-014, Life Technologies, Inc.) in blocking solution for 2 h at room temperature. After washing, the antigen-antibody complexes were visualized by chemiluminescence (SuperSignal, Pierce) and exposed on film (X-OMAT, Eastman Kodak Co.). Signal transduction kinases were analyzed by immunoblotting by Kinexus Bioinformatics (Vancouver, Canada).

Protein Kinase Assays—Cdc2 was immunoprecipitated, and its kinase activity was assayed as described in Ref. 52. ATM and ATR were immunoprecipitated from human lymphoblastoid BT cells using antibody Ab-3 and Ab-1 (Oncogene Research Products) respectively, and their kinase activities assayed using 10 mM HEPES, pH 7.5, 50 mM β-glycerophosphate, 50 mM NaCl, 10 mM MnCl2, 10 μM ATP, and 5 μCi [32P]ATP per assay as described in Ref. 53. DNA-PK catalytic subunit and Ku70/80 were purified from human placenta, reconstituted to give a 1:1 molar ratio of DNA-PK catalytic subunit to Ku, and assayed as described in Ref. 54. The activity of recombinant glutathione S-transferase-tagged Chk2 was assayed in 50 mM Tris, pH 8.0, 50 mM KCl, 5% glycerol, 10 mM MnCl2, 0.5 μg of recombinant His-tagged PHAS-1 (Stratagene), 0.1 μg of Chk2, and 10 μM ATP containing 5 μCi [32P]ATP. Reactions were for 30 min at 30 °C. The kinase activity of glutathione S-transferase-tagged baculovirus-expressed human Chk1 was assayed as described in Ref. 55 using the peptide GLYRAPSMPPNLRK (derived from residues 210–223 of human Cdc25C containing an S214A substitution) as a substrate.

Results

Isolation and Identification of DBH—A cell-based assay for G2 checkpoint inhibition (29) was used to screen several thousand crude organic extracts from marine invertebrates. An extract from a sponge collected in Papua New Guinea showed activity. A compound with strong G2 checkpoint inhibitory activity was isolated by chromatographic procedures (see “Experimental Procedures”) using the cell-based assay to direct purification. The active compound was identified as DBH (Fig. 1) by analysis of its mass spectrometry and NMR data and comparison with published values (57). DBH was identified first in 1980 as a yellow compound from the sponge Phakellia flabelata (57). The extract also contained minor amounts of the structurally related compounds hynemialdisine, debromoxinohydanon, and debromopyrrolococlam (Fig. 1).

Activity Profile of DBH and Analogos—The activity profile of DBH as a G2 checkpoint inhibitor is shown in Fig. 2A. MCF-7 mp53 cells arrested in the G2 phase after exposure to ionizing radiation were incubated with DBH for 8 h in the presence of nocodazole to trap cells in mitosis. The number of mitotic cells was counted by microscopy. DBH showed dose-dependent G2 checkpoint inhibition with an IC50 of 8 μM and maximal activity at 40 μM. Higher concentrations caused a decrease in activity, a phenomenon also seen with other checkpoint inhibitors (29, 35) and believed to be caused by the inhibition of enzymatic activities required for cell cycle progression (45).

We next determined the effect of DBH on cell proliferation. Cyclohexyl cells were incubated with DBH for 20 h, the compound was washed away, and the cells were allowed to grow. Cell proliferation was determined after 3 days using the MTT assay. DBH inhibited cell proliferation but not potently, with an IC50 of 25 μM (Fig. 2B).

Hymenialdisine was active also as a checkpoint inhibitor with an IC50 of 6 μM (Table I). Debromoxinohydanon and...
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Debromohymenialdisine, which were present also in the sponge extract, and the related compounds 2-aminoimidazole and 2-amino-4,5-imidazole-dicarbonitrile obtained from commercial sources showed no activity at all concentrations tested (Table I). Only DBH was isolated in sufficient quantities for the additional studies described below.

Activation of Cdc2 Kinase by DBH—When cells are arrested in the G2 phase by DNA damage, Cdc2 kinase is maintained in an inactive state by phosphorylation of Thr and Tyr residues in its ATP-binding site. Entry into mitosis requires the dephosphorylation of these residues by Cdc25 protein phosphatases. We first determined whether DBH overcomes G2 arrest by affecting the phosphorylation and the protein kinase activity of Cdc2.

Extracts were prepared from cells arrested in mitosis by treatment with nocodazole, from cells arrested in G2 phase after exposure to ionizing radiation, or from cells arrested in G2 phase and then treated with 40 μM DBH for up to 6 h. Cellular proteins were separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting using a Cdc2 antibody. Depending on its inhibitory phosphorylation state, Cdc2 may be detected as slow migrating bands corresponding to phosphorylated inactive kinase and a faster migrating band corresponding to hypophosphorylated active Cdc2. As shown in Fig. 3A, cells arrested in mitosis with nocodazole had no detectable phosphorylated inhibited Cdc2, whereas cells arrested in G2 phase after irradiation contained phosphorylated Cdc2. Cells arrested in G2 and incubated with DBH showed a time-dependent decrease in the amount of phosphorylated Cdc2, with the slowest migrating band essentially becoming undetectable by 6 h. The well characterized G2 checkpoint inhibitor caffeine also caused a decrease of phosphorylated Cdc2 (Fig. 3A).

We next examined the activity of immunoprecipitated Cdc2 using histone H1 as a substrate. Cdc2 activity was high in cells arrested in mitosis and very low in cells arrested in G2 phase by irradiation (Fig. 3B). DBH caused a concentration-dependent activation of Cdc2. DBH at 4 and 40 μM activated Cdc2 to levels activated by 0.2 and 2 mM caffeine, respectively (Fig. 3B). Taken together, these results indicate that DBH overcomes G2 arrest by interfering with the inhibitory phosphorylation of Cdc2 kinase and causing its activation.

Inhibition of Chk1 and Chk2 by DBH—Hymenialdisine was shown recently to inhibit several protein kinases in vitro (58). However, this study did not test kinases involved in the G2 checkpoint. The checkpoint inhibitor 7-hydroxystaurosporine inhibits Chk1 but not Chk2 (43, 44), and caffeine inhibits ATM and ATR (12, 46–48). Therefore, we next examined whether DBH inhibits checkpoint kinases.

The effect of DBH on the protein kinase activity of purified DNA-PK was determined using a synthetic peptide derived from p53 as described previously (59). More than 70% of the protein kinase activity of DNA-PK was retained in the presence of 20 μM DBH, whereas the known DNA-PK inhibitor wortmannin (1 μM) effectively abolished DNA-PK activity (Fig. 4A). To determine the effect of DBH on the protein kinase activity of ATM and ATR, the proteins were immunoprecipitated from human lymphoblastoid cells and assayed in the presence of different concentrations of DBH. DBH did not cause significant inhibition of ATM and ATR in these assays, suggesting that it does not act on phosphatidylinositol 3-kinase-like enzymes (Fig. 4B).

The effect of DBH on Chk2 activity was also measured. As shown in Fig. 5, DBH was a potent inhibitor of Chk2 kinase activity with an IC50 of 3.5 μM. DBH also inhibited autophosphorylation of Chk2 (data not shown). The activity of human recombinant Chk1 was assayed using a Cdc25C-derived peptide as a substrate (55) in the presence of different concentrations of DBH. DBH inhibited Chk1 with an IC50 of 3 μM (Fig. 6). To determine whether DBH inhibits the activity of Chk1 by competing with ATP, Chk1 activity was assayed with varying concentrations of ATP and with different concentrations of DBH. As shown in Fig. 6 (inset), DBH inhibits Chk1 competitively with respect to ATP.

Effect of DBH on the Intracellular Localization of Cell Cycle Regulatory Proteins—In the yeast Schizosaccharomyces pombe, the Chk1 protein kinase is essential for DNA damage-induced G2 arrest (60). Chk1 can phosphorylate Cdc25 (13), creating a binding site for 14-3-3 proteins. The Cdc25-14-3-3 complexes localize to the cytoplasm, in which they are separated from the nuclear pools of Cdc2 kinase. This provides an attractive model for checkpoint arrest in organisms such as fission yeast, in which Cdc2 kinase is exclusively nuclear and mitosis is closed.

Table I

| Compound                          | G2 checkpoint inhibition IC50 μM |
|-----------------------------------|----------------------------------|
| Debromohymenialdisine             | 8 ± 4                            |
| Hymenialdisine                    | 6 ± 3                            |
| Debromoxinolactam                 | >200                             |
| Debromopyrrolactam                | >200                             |
| 2-Aminoimidazole                  | >10,000                          |
| 2-Amino-4,5-imidazole-dicarbonitrile | >10,000                      |

FIG. 2. Inhibition of G2 checkpoint and of cell proliferation by DBH. A, MCF-7 mp53 cells arrested in G2 phase by ionizing radiation were treated with different concentrations of DBH together with nocodazole for 8 h. G2 checkpoint inhibition was determined by counting the number of mitotic cells by microscopy. B, MCF-7 mp33 cells were exposed to different concentrations of DBH for 20 h, and cell proliferation was assayed 3 days later as described under “Experimental Procedures.”

FIG. 3. Effect of DBH on the phosphorylation and activity of Cdc2 kinase. A, Cdc2 immunoblot from cells arrested in the G2 phase and from cells arrested in the G2 phase and exposed to 40 μM DBH or 2 mM caffeine (caf) for 2, 4, or 6 h. B, histone H1 kinase activity of Cdc2 immunoprecipitated from cells arrested in the G2 or M phase or from G2-arrested cells exposed to DBH or caffeine for 4 h.

FIG. 4. A, DBH inhibited Chk1 activity with an IC50 of 3 μM. B, DBH inhibited Chk2 activity with an IC50 of 3.5 μM.
However, in human cells at least some Cdc2-cyclin B and Cdc25C localizes to the cytoplasm in interphase (15), and it is not clear how phosphorylation-induced retention of Cdc25C-3 complexes in the cytoplasm could contribute to G2 arrest. Cdc25C and cyclin B enter the nucleus just prior to mitosis.

One possibility is that 14-3-3 binding to Cdc25C prevents this event. Because of the possible importance of cellular compartmentalization in checkpoint arrest, we examined the effects of irradiation and DBH treatment on the intracellular localization of a variety of cell cycle regulatory proteins implicated in the control of the G2/M transition.

Immunofluorescence microscopy was performed on cycling MCF-7 mp53 cells, G2-arrested cells, or G2-arrested cells treated for 3 h with 40 \( \mu M \) DBH. We observed no changes in the cellular localization of Cdc2, Cdc25C, cyclin A, cyclin B, Cdk2, Cdk25B, Wee1, and 14-3-3 (data not shown) between cycling cells and irradiated G2-arrested cells. Although subtle changes in cellular distribution may have been missed using this technique, these data indicate that in this cell line, G2 arrest is not accompanied by a major change in the nuclear versus cytoplasmic distribution of these proteins. DBH treatment of G2-arrested cells caused no significant changes in the distribution of Cdc2, Cdc25C, cyclin A, cyclin B, Cdk2, Cdk25B, Wee1, and 14-3-3 in cells that did not yet show morphological evidence of having entered mitosis (data not shown). Similarly, the distribution of these proteins in cells forced to enter mitosis by DBH was not different from that of cells in a normal mitosis (data not shown).

**Effect of DBH on Other Signal Transduction Kinases**—Signal transduction pathways are controlled typically by kinase phosphorylation cascades. Many of these kinases undergo mobility changes in SDS-polyacrylamide gel electrophoresis after phosphorylation, reflecting the activation state of the pathways. To determine whether DBH affects many or a few kinases in vivo, its effects on the abundance and mobility of 24 kinases were evaluated. Cycling MCF-7 mp53 cells and G2-arrested cells treated or not treated with 40 \( \mu M \) DBH for 2 h were harvested. Cell extracts were prepared in buffer containing phosphatase and protease inhibitors, and proteins were separated using SDS-polyacrylamide gel electrophoresis. The proteins then were subjected to immunoblotting with a panel of antibodies that recognize kinases that undergo mobility shift when activated or inhibited by phosphorylation: CaMK4, Cdk2, CK1\( \alpha \), CK2\( \alpha \), Erk1, Erk2, p38 Hog mitogen-activated protein kinase, Mek1, Mek2, Mek3, Mek4, Mek5, Mekk3, Pim1, PKB\( \alpha \), PKC\( \beta \), PKC\( \epsilon \), PKC\( \gamma \), Rek1, p70S6k, p46 SAPK, p54 SAPK, and Tak1. All the antibodies revealed clear bands at the expected molecular masses of their respective kinases, but none showed changes in abundance or mobility after treatment with DBH (data not shown). This shows that DBH is not a broad spectrum protein kinase inhibitor in vivo.

**DISCUSSION**

This study identifies DBH and hymenialdisine as new G2 checkpoint inhibitors. These compounds show no obvious structural resemblance to previously described checkpoint inhibitors. The bulky bromine substituent at position 2 in hymenialdisine that is absent in DBH does not influence G2 checkpoint inhibition significantly, the two compounds having IC50 values of 6 and 8 \( \mu M \), respectively (Table I). Both the pyrrololactam and the aminoimidazole moieties are required because 2-ami-
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We propose that the checkpoint kinases Chk1 and Chk2 are the targets of DBH involved in the G2 checkpoint. Preliminary data indicate that DBH has little or no effect on the activity of Wee1 in vitro at concentrations up to 10 μM (data not shown). However, Myt1 cannot be excluded as a target because it was not tested. The concentration of DBH required to inhibit Chk1 and Chk2 in vitro (IC50 = 3 and 3.5 μM, respectively) is close to that required for inhibition of the checkpoint in vivo (IC50 = 8 μM). Both kinases probably cooperate in maintaining Cdc25C phosphorylated at Ser-216 after DNA damage, and it seems likely that DBH owes its high efficacy as a checkpoint inhibitor to inhibition of both kinases. Structural and functional redundancy of kinases is common in mammalian signal transduction pathways. It may be generally the case that agents with broader specificity will have more impact on complex signaling pathways than highly selective agents.

Irradiation and DBH treatment caused no noticeable alteration of the cellular distribution of the major cell cycle regulatory proteins Cdc2, Cdc25C, cyclin A, cyclin B, Cdk2, Cdc25B, Wee1, and 14-3-3 in MCF-7mp53 cells. In addition, we saw no evidence of irradiation causing a physical separation of Cdc2-

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