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Irofulven (6-hydroxymethylacylfulvene, HMAF, MGI 114) is one of a new class of anticancer agents that are semisynthetic derivatives of the mushroom toxin illudin S. Preclinical studies and clinical trials have demonstrated that irofulven is effective against several tumor types. Mechanisms of action studies indicate that irofulven induces DNA damage, MAPK activation, and apoptosis. In this study we found that in ovarian cancer cells, CHK2 kinase is activated by irofulven while CHK1 kinase is not activated even when treated at higher concentrations of the drug. By using GM00847 human fibroblast expressing tetracycline-controlled, FLAG-tagged kinase-dead ATR (ATR.kd), it was demonstrated that ATR kinase does not play a major role in irofulven-induced CHK2 activation. Results from human fibroblasts proficient or deficient in ATM function (GM00637 and GM00490) indicated that CHK2 activation by irofulven is mediated by the upstream ATM kinase. Phosphorylation of ATM on Ser1981, which is critical for kinase activation, was observed in ovarian cancer cell lines treated with irofulven. RNA interference experiments confirmed that CHK2 activation was inhibited after introducing siRNA for ATM. Finally, experiments done with human colon cancer cell line HCT116 and its isogenic CHK2 knockout derivative, and experiments done by expressing kinase-dead CHK2 in an ovarian cancer cell line demonstrated that CHK2 activation contributes to irofulven-induced S phase arrest. In addition, it was shown that NBS1, SMC1, and p53 were phosphorylated in an ATM-dependent manner, and p53 phosphorylation on serine 20 is dependent on CHK2 after irofulven treatment. In summary, we found that the anticancer agent, irofulven, activates the ATM-CHK2 DNA damage-signaling pathway, and CHK2 activation contributes to S phase cell cycle arrest induced by irofulven.

Irofulven (6-hydroxymethylacylfulvene, HMAF, MGI 114) is one of a new class of anticancer agents that are analogs of mushroom-derived illudin toxins. Preclinical studies and clinical trials have demonstrated that irofulven is effective against several tumor types (1–17). Studies of mechanisms of irofulven action suggest that it induces DNA damage, MAP kinase activation and apoptosis (18–20). It is also suggested that irofulven-elicited DNA lesions are mainly repaired by transcription-coupled nucleotide excision repair (TC-NER) (21).

In response to DNA damage, the cell evokes signal transduction pathways to arrest at G1/S, S, or G2/M checkpoints, allowing time to deal with the insult (22, 23). It has been well documented that DNA damage activates ATM (ataxia telangiectasia-mutated), and/or ATR (ATM-RAD3-related) kinases, two apical protein kinases of the DNA damage response pathways. ATM and ATR phosphorylate downstream effector kinases, CHK1 and CHK2. It is generally believed that ATM is the kinase mainly responding to ionizing radiation (IR)-induced DNA double strand breaks, while ATR responds to the formation of DNA adducts and stalled replication induced by UV, genotoxic drugs, and radiation (23–29).

ATM phosphorylates NBS1 on Ser343 and activates its function in forming the MRE11-RAD50-NBS1 complex and S phase checkpoint control (30–32). ATM phosphorylates SMC1 on Ser237 and Ser266. Activated SMC1 plays a critical role in S phase checkpoint control and radiosensitivity (33, 34). ATM also phosphorylates MDM2 on Ser395, indirectly regulating p53 activity (35). Both ATM and CHK2 phosphorylate BRCA1 (36–38). BRCA1 plays an important role in S and G2/M checkpoint control (39–41).

ATM phosphorylates CHK2 on Thr68 leading to CHK2 kinase activation (42–46), while both ATM and ATR phosphorylate CHK1 on Ser317 and Ser345 resulting in its activation (29, 47–49). ATM and ATR phosphorylate p53 on Ser15 (23, 50–53), and CHK1 and CHK2 phosphorylate p53 on Ser20 (54–59), leading to its accumulation and activation. Activation of p53 initiates cell cycle arrest and DNA repair-related genes such as p21, GADD45, and 14-3-3-σ, and leads to G1 and G2 arrest (22, 23, 54–56, 60, 61). Activation of p53 also regulates the expression of a plethora of apoptosis-related genes resulting in p53-dependent apoptosis (62–64). Activation of CHK1 and CHK2 also regulates S phase by phosphorylating CDC25A (65–72), or the G2/M transition by phosphorylating CDC25C (29, 42, 68, 71, 73–76). However, controversy exists regarding the role of CHK2 in cell cycle regulation and p53 phosphorylation. Recent studies indicate that CHK2 is dispensable in radiation-induced G1 and G2/M arrest, and CHK1 and CHK2 are unlikely to be the regulators of p53 (77–79). It has also been shown that cells thioisocyanate; MAPK, mitogen-activated protein kinase; GFP, green fluorescent protein.
Irofulven Activates CHK2 via ATM

Irofulven Activates CHK2 Kinase in Ovarian Cancer Cells—To assess irofulven cytotoxicity, ovarian cancer cell lines were treated with different concentrations of irofulven for 1 h, then the drug was removed, and cell proliferation assay was performed. The IC₅₀ concentrations obtained for ovarian cancer cell lines (A2780, A2780/CP70, CAOV3, SKOV3, and OVCAR3) ranged from 0.7 to 2.3 μM.

In response to DNA damage, CHK2 kinase is phosphorylated at Thr⁶⁸, which is critical for CHK2 activation (42–44, 46, 83). To investigate the DNA damage response pathway that might be activated by irofulven-induced DNA damage, ovarian cancer cells were treated with the 1 × IC₅₀ concentration of irofulven for 1 h, and incubated for 24 h after drug treatment. Western blot assay was performed with antibody recognizing the phosphorylated form of CHK2 on Thr⁶⁸. As indicated in Fig. 1A, CHK2 kinase was activated by irofulven in all ovarian cancer cell lines. The activation of CHK2 kinase by irofulven was time and dose-dependent (Fig. 1, B and C).

CHK1 Kinase Is Not Activated by Irofulven—Because CHK1 kinase primarily responds to UV and drug-induced DNA damage (23–29), it was critical to test whether CHK1 was activated by irofulven-induced DNA damage. Western blot assay was performed with antibody recognizing phosphorylated CHK1 on Ser⁴⁴⁵. As shown in Fig. 2, CHK1 was not activated by irofulven in all four cell lines tested, even when cells were treated with 3 × IC₅₀ concentrations, a treatment resulting in ~90% cell killing. A2780 treated with 50 J/m² of UV light served as positive control for antibody against phosphorylated CHK1 (Fig. 2).

Next, we examined whether CHK2 was activated in the absence of CHK2 expression. Isogenic human colon cancer cell line HCT116 and its CHK2 knockout derivative (78) were treated with 1 × IC₅₀ concentration of irofulven for 1 h followed
Irofulven Activates CHK2 via ATM

by an additional 12- or 24-h incubation in drug-free media. Western blot analyses were performed with antibodies against phosphorylated CHK1 on Ser345, CHK1, CHK2, and actin. As shown in Fig. 3A, CHK1 phosphorylation was not detected in CHK2+/+ or CHK2−/− cells. A2780 treated with 50 J/m² of UV light served as positive control for CHK1 activation. The Western blot for CHK2 confirmed that CHK2 expression is absent in CHK2−/− cells (Fig. 3A). Further, to demonstrate that the CHK1 activation pathway is intact in these cells, parental HCT116 (CHK2+/+) cells were treated with 50 J/m² of UV light, and Western blot results indicated that the pathway leading to CHK1 phosphorylation is indeed intact (Fig. 3B). This is consistent with the finding that CHK1 can be activated when these cells were treated with ionizing radiation (78).

**CHK2 Activation by Irofulven Is Not Mediated by ATR**—Both ATM and ATR have been reported to activate CHK2 in response to DNA damage (28, 42–46, 84, 85). To determine the possible involvement of ATR in irofulven-induced CHK2 activation, GM00847 human fibroblasts expressing tetracycline-controlled, FLAG-tagged kinase-dead ATR (ATR.kd) (81) were used. Both doxycycline-induced and un-induced cells were treated with irofulven. As shown in Fig. 4A, the FLAG-tagged, kinase-dead ATR was strongly induced by 1.5 μg/ml of doxycycline for 48 h as determined by Western blot with anti-FLAG antibody. However, under the same conditions, CHK2 activation by irofulven (8 μM, 1 h treatment followed by 12 h of incubation) was apparent. There actually was some increase of phosphorylated CHK2 in cells expressing kinase-dead ATR after irofulven treatment (Fig. 4A). To confirm that the doxycycline-induced kinase-dead ATR was functional, the doxycycline-treated and untreated cells were exposed to 50 J/m² of UV light. As shown in Fig. 4B, CHK1 kinase was strongly activated by UV in un-induced cells, but the induced kinase-dead ATR greatly blocked CHK1 activation by UV irradiation, indicating that ATR.kd was functional.

**CHK2 Activation by Irofulven Is Dependent on ATM**—To determine the role that ATM might play in irofulven-induced CHK2 activation, Western blot assay was carried out with cellular extracts from ATM-deficient and proficient cell lines treated with irofulven. As shown in Fig. 5A, CHK2 was activated by irofulven in a concentration-dependent manner in the SV40-transformed normal human fibroblast GM00637. In contrast, under the same irofulven treatment conditions, CHK2 was not activated in SV40-transformed human AT (ataxia telangiectasia) fibroblast GM05849, and its parental non-transformed AT fibroblast, GM00637, in which CHK2 was not activated in SV40-transformed human AT (ataxia telangiectasia) fibroblast GM05849, and its parental non-transformed AT fibroblast, GM00637, which are known to be deficient in ATM function (Fig. 5B). As a positive control, the CHK2 activation was observed in the ATM-complemented AT fibroblast (AT22JE-T-pEBST-YZ5) (80) (Fig. 5B). Taken together, these results indicate that the CHK2 activation by irofulven is dependent on the ATM status.

DNA damage induces rapid autophosphorylation of ATM on serine 1981. This ATM autophosphorylation causes dimer dissociation and initiates ATM kinase activity (86). To further study the involvement of ATM kinase activation in irofulven-induced CHK2 activation, A2780 and CAOV3 ovarian cancer cells were treated with irofulven for 1 h followed by 24 h of

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**Fig. 1.** CHK2 activation by irofulven in ovarian cancer cell lines. Antibody recognizing the phosphorylated form of CHK2 kinase on Thr68 was used to determine the activated CHK2. Blots for CHK2 and actin served as loading control. A, cells were treated with 1× IC₅₀ concentration of irofulven for 1 h followed by additional 24 h of incubation. B, cells were treated as above followed by different incubation time. C, cells were treated with 1× or 3× IC₅₀ concentration of irofulven for 1 h followed by additional 24 h of incubation.

**Fig. 2.** CHK1 is not activated by irofulven. Ovarian cancer cell lines were treated with 1× or 3× IC₅₀ concentration of irofulven for 1 h followed by additional 24 h of incubation. Western blot was performed with antibody recognizing phosphorylated CHK1 on Ser345. Extracts from UV (50 J/m²)-treated A2780 cells were loaded as positive control for CHK1 activation. Blots for CHK1 and actin served as loading control.

**Fig. 3.** CHK1 is not activated by irofulven in the absence of CHK2 expression. A, human colon cancer cell line HCT116 and its CHK2 knockout derivative were treated with 1× IC₅₀ concentration of irofulven for 1 h followed by an additional 12 or 24 h of incubation. CHK1 activation was observed by Western blot with antibody recognizing the phosphorylated form of CHK1 on Ser345. Western blots for CHK1 and actin served as loading control. The blot for CHK2 indicated the CHK2 status in parental and CHK2 knockout cells. A2780 treated with 50 J/m² of UV light served as positive control for antibody against phosphorylated CHK1 on Ser345. B, parental HCT116 (CHK2+/+) cells were treated with 50 J/m² of UV light, CHK1 activation was determined by Western blot with anti-phospho-CHK1 antibody, and the blots for CHK1 and actin were shown as the loading control.
Irofulven Activates CHK2 via ATM

**Experimental Procedures.**

**ATM kinase phosphorylation on Ser 1981 in both A2780 and CAOV3 incubation.** ATR kinase-dead ATR (ATR.kd) was induced with 1.5 μg/ml of doxycycline for 48 h. The induced and un-induced cells were then treated with 8 μM of irofulven for 1 h followed by 12 h of post-treatment incubation. A, ATR.kd induction was shown on the top panel by Western blot with anti-FLAG antibody. The nonspecific band (n.s.) was shown as the loading control for FLAG-ATR.kd induction. CHK2 activation was shown by Western blot with anti-phospho-CHK2 antibody on the bottom panel. The blot for CHK2 was shown as the loading control. B, induced and un-induced GM00847-ATR.kd cells were treated with 50 J/m² of UV light. CHK1 activation was determined by Western blot with anti-FLAG antibody. The nonspecific band (n.s.) was shown as the loading control for FLAG-ATR.kd induction. CHK2 activation was determined by Western blot with anti-phospho-CHK2 antibody. The nonspecific band (n.s.) was shown as the loading control.

**Fig. 4.** The role of ATR in CHK2 activation by irofulven. GM00847 human fibroblast expressing tetracycline-controlled, FLAG-tagged kinase-dead ATR (ATR.kd) was induced with 1.5 μg/ml of doxycycline for 48 h. The induced and un-induced cells were then treated with 8 μM of irofulven for 1 h followed by 12 h of post-treatment incubation. A, ATR.kd induction was shown on the top panel by Western blot with anti-FLAG antibody. The nonspecific band (n.s.) was shown as the loading control for FLAG-ATR.kd induction. CHK2 activation was shown by Western blot with anti-phospho-CHK2 antibody on the bottom panel. The blot for CHK2 was shown as the loading control. B, induced and un-induced GM00847-ATR.kd cells were treated with 50 J/m² of UV light. CHK1 activation was determined by Western blot with anti-FLAG antibody. The nonspecific band (n.s.) was shown as the loading control for FLAG-ATR.kd induction. CHK2 activation was determined by Western blot with anti-phospho-CHK2 antibody. The nonspecific band (n.s.) was shown as the loading control.

**Fig. 5.** The role of ATM in CHK2 activation by irofulven. A, SV40-transformed human normal fibroblast GM00637 was treated with different concentrations of irofulven as indicated for 1 h followed by 24 h of drug-free incubation. CHK2 activation was determined by Western blot with anti-phospho-CHK2 antibody. Western blots for CHK2 and actin were shown as the loading control. B, SV40-transformed human AT (ataxia telangiectasia) fibroblast, GM05849, its parental non-transformed AT fibroblast, GM05823, and the ATM-complemented AT fibroblast (AT22IJE-T-pEBS7-YZ5) were treated with irofulven. The CHK2 activation was determined by Western blot with anti-phospho-CHK2 antibody, the nonspecific band (n.s.) was shown as the loading control.

**Fig. 6.** ATM is phosphorylated following irofulven treatment in ovarian cancer cells and inhibition of ATM expression blocks irofulven-induced CHK2 activation. A, ovarian cancer cell lines A2780 and CAOV3 were treated with their respective 1 × IC₅₀ concentration of irofulven for 1 h followed by 24 h of drug-free incubation. The phosphorylation of ATM kinase on Ser1981 was determined by Western blot with anti-phospho-Ser1981-ATM antibody. B, A2780 cells were transfected with small interfering RNAs for GFP (siGFP) and ATM (siATM) as described under "Experimental Procedures." ATM protein level and CHK2 activation were determined by Western blot with anti-ATM and anti-phospho-CHK2 antibodies. Western blots for CHK2 and actin were used as the loading control.

Irofulven treatment was greatly attenuated in cells transfected with siATM (Fig. 6B).

**ATM and CHK2 Target Proteins Activated by Irofulven—**

In response to ionizing radiation, ATM phosphorylates NBS1 on Ser343 (30–32), SMC1 on Ser957, and BRCA1 (33, 34), p53 on Ser15 (52, 53, 87, 88). ATM and CHK2 phosphorylate BRCA1 on multiple serine sites (36–38). CHK2 phosphorylates p53 on Ser392 and stabilizes p53 (54, 56, 59, 89).

In an effort to further understand irofulven-induced activation of ATM and CHK2, the activation of ATM and CHK2 target proteins was tested. Ovarian cancer cell lines A2780, CAOV3, SKOV3, and OVCAR3 were treated with irofulven, Western blot results demonstrated that NBS1 was phosphorylated on Ser343 in SKOV3 and OVCAR3 cells, but not in A2780 and CAOV3 cells as determined with antibody recognizing phosphorylated NBS1. When the NBS1 protein expression levels were compared, it was shown that much less NBS1 was expressed in A2780 and CAOV3 cells than in SKOV3 and OVCAR3 cells (Fig. 7A). Further, Western blot results also demonstrated that SMC1 was phosphorylated in CAOV3, SKOV3 and OVCAR3 cells as determined by antibody recognizing phosphorylated SMC1 on Ser957. Phosphorylation of p53 on Ser15 was observed in A2780 and CAOV3 cells (Fig. 7A). In the three other cell lines, p53 is known to be mutated (91).

To determine the dependence of NBS1, SMC1, and p53 activation on ATM after irofulven treatment, normal human fibroblast GM00637 and AT fibroblast GM05849 were treated with irofulven and Western blot results indicated that more NBS1 was phosphorylated on Ser343 and band-shifted in ATM wild-type GM00637 cells than in AT cells, GM05849. Similarly, more SMC1 phosphorylation on Ser957 and p53 phosphorylation on Ser15 were observed in ATM wild-type cells than in AT cells (Fig. 7B).

In summary, NBS1, SMC1, and p53 were activated by irofulven both in ovarian cancer cell lines and in normal human fibroblasts, and this activation is dependent on ATM.

CHK2 phosphorylates p53 on Ser392 and this phosphorylation event stabilizes the p53 protein (54–56, 59, 68). However, recent studies suggest that it is unlikely that CHK2 regulates p53 (78, 79). To determine whether irofulven-induced CHK2

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**ATM is phosphorylated following irofulven treatment in ovarian cancer cells and inhibition of ATM expression blocks irofulven-induced CHK2 activation.** A, ovarian cancer cell lines A2780 and CAOV3 were treated with their respective 1 × IC₅₀ concentration of irofulven for 1 h followed by 24 h of drug-free incubation. The phosphorylation of ATM kinase on Ser1981 was determined by Western blot with anti-phospho-Ser1981-ATM antibody. B, A2780 cells were transfected with small interfering RNAs for GFP (siGFP) and ATM (siATM) as described under "Experimental Procedures." ATM protein level and CHK2 activation were determined by Western blot with anti-ATM and anti-phospho-CHK2 antibodies. Western blots for CHK2 and actin were used as the loading control.
activation has any effect on p53 phosphorylation on Ser\(^{20}\), antibody recognizing phosphorylated p53 on Ser\(^{20}\) was used in Western blot analysis. In A2780 (wild-type p53) and CAOV3 (mutated p53) cells after irofulven treatment, p53 phosphorylation and protein accumulation were observed (Fig. 7C). To assess whether p53 phosphorylation on Ser\(^{20}\) is dependent on CHK2, the human colon cancer cell line HCT116 and its CHK2 knockout derivative (78) were treated with irofulven and Western blot results indicated that increased phosphorylation of p53 on Ser\(^{20}\), and greater p53 protein accumulation were observed in parental HCT116 cells compared with CHK2 knockout cells, suggesting this phosphorylation event is dependent on CHK2 status (Fig. 7D).

BRCA1 phosphorylation was also determined by Western blot in HCT116 cells and its CHK2 knockout cells treated with irofulven, but no difference on BRCA1 phosphorylation was observed (data not shown).

Taken together, in response to irofulven-induced DNA damage, p53 was phosphorylated on serine 20 in a CHK2-dependent manner.

**CHK2 Activation by Irofulven Contributes to S Phase Arrest**—Many studies have linked CHK2 activation to G\(_1\), S or G\(_2\)/M phase arrest, respectively (42, 54–56, 65–67, 73, 74). To understand the possible role that irofulven-induced CHK2 activation might play in cell cycle arrest, we examined the isogenic human colon cancer cell line HCT116 and its CHK2 knockout derivative (78) after irofulven treatment. These paired cell lines were treated with 1\( \times \) IC\(_{50}\) concentration of irofulven for 1 h followed by additional 12 or 24 h of incubation in drug-free media. Western blot analysis indicated that CHK2 was only expressed and activated in parental HCT116 (CHK2/\(+/+\)) cells (Fig. 8A). To determine the effect of CHK2 activation on cell cycle arrest, the CHK2/\(+/+\) and CHK2/\(−/−\) cells were pulse-labeled with BrdU before drug treatment, harvested at different time points and stained with FITC-conjugated anti-BrdU antibody and propidium iodide. FACS analysis for BrdU-positive cells indicated that at time zero, in the untreated control groups, there were 84% of CHK2/\(+/+\) cells in S phase compared with 75% of CHK2/\(−/−\) cells in S phase.

Three hours after the drug treatment, there were 4-fold more CHK2/\(+/+\) cells arrested at S phase than CHK2/\(−/−\) cells. This trend was maintained throughout the 24-h period after drug removal (Fig. 8B). Similarly, when comparing the S phase ratio of irofulven-treated over untreated in each cell line, it was clearly shown that there were more cells arrested at S phase in CHK2/\(+/+\) than in CHK2/\(−/−\) cells (Fig. 8C).

In response to IR-induced DNA damage, ATM and CHK2 phosphorylate CDC25A on serine 123, leading to its ubiquitination and degradation, and consequently resulting in S phase arrest (65, 66). To further confirm the results obtained with CHK2 knockout cell lines, the ovarian cancer cell line CAOV3 was first treated with irofulven and CDC25A protein level was determined by Western blot. 12 h after irofulven treatment, CDC25A was degraded. Addition of the proteasome inhibitor, LLnL, could block this degradation, indicating cells were arrested at S phase at this time point (Fig. 9A). To further study the role that CHK2 activation plays in irofulven-induced S phase arrest, CAOV3 cells were transfected with vector or HA-tagged kinase-dead CHK2 (CHK2.kd) (82). Cells were then treated with irofulven and stained with FITC-conjugated anti-HA antibody and propidium iodide. Results of FACS analysis performed 12 h post-drug treatment were consistent with CDC25A degradation studies and indicated that in vector-transfected cells, ~38% of untreated cells were in S phase, whereas ~45% of irofulven-treated cells were in S phase (Fig. 9, B and C). Among the CHK2.kd-transfected cells, the total cell population presented a similar cell cycle distribution pattern as vector-transfected cells after irofulven treatment. But when cells were gated for only FITC-positive (CHK2.kd-transfected) cells, after irofulven treatment, the number of cells in S phase was decreased by 20% (Fig. 9, B and C), indicating that introduction of kinase-dead CHK2 inhibited irofulven-induced S phase arrest.

Taken together, it was concluded that CHK2 activation by irofulven contributes to S phase arrest.
Fig. 8. Irofulven-induced CHK2 activation contributes to S phase arrest. A, a human colon cancer cell line HCT116 and its CHK2 knockout derivative were treated with 1× IC_{50} concentration of irofulven for 1 h followed by additional 12 or 24 h of incubation, CHK2 activation was observed by Western blot with antibody recognizing the phosphorylated form of CHK2 kinase on Thr^{68}. Western blots for CHK2 and actin served as loading control. B and C, HCT116 CHK2+/+ and CHK2−/− cells were pulse-labeled with 10 μM of BrdU for 1 h followed by 1-h drug treatment at 1× IC_{50} concentration. Untreated and treated cells were harvested at different time point and stained with FITC-conjugated anti-BrdU antibody and propidium iodide. Cells were then applied to FACS analysis. The BrdU-positive cells were gated and histograms of DNA content versus cell counts (30,000 events) were obtained. The percentage of each cell cycle phase was analyzed by ModFit v3.0 software. S phase percentage of irofulven-treated over untreated for CHK2+/+ and CHK2−/− cells was shown in B. The ratio of S phase percentage of irofulven-treated over untreated for CHK2+/+ and CHK2−/− cells was shown in C.
DISCUSSION

Irofulven is a novel semi-synthetic derivative of the mushroom toxin, illudin S, which has demonstrated antitumor activity against prostate, pancreatic, and ovarian cancer in clinical trials. The mechanism of irofulven action involves several biological processes including DNA damage, MAPK signaling, cell cycle arrest, and caspase-dependent apoptosis. The chemical structure and nature of irofulven-elicited DNA lesions are currently unknown. By using paired cell lines proficient and deficient for ATM, ATR or CHK2 function, and by using RNA interference techniques and transfection of dominant-negative CHK2, we show that irofulven induces ATM-dependent CHK2 activation leading to S phase arrest, a pathway that has primarily been characterized in response to ionizing radiation-induced DNA double strand breaks (23–29). In contrast, the ATR and CHK1 pathway, which mainly responds to drug and UV-induced DNA lesions (23–29), does not play an important role in irofulven-induced DNA damage response. In addition, we also show that ATM target proteins NBS1, SMC1, and p53 were also phosphorylated in an ATM-dependent manner upon irofulven treatment, and p53 phosphorylation on serine 20 induced by irofulven is dependent on CHK2 status. These novel

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observations may aid in the further elucidation of the molecular mechanisms of action of irofulven, and in the potential design of combined therapy with radiation, S phase abrogators, or inhibitors of the ATM-CHK2 signaling pathway.

CHK2 has been shown to be involved in DNA repair process at stalled replication forks by interacting with ATR, p53, and BRCA1 (28, 92–94). A recent study done in fission yeast demonstrated that Cds1 (CHK2) interacts with Rad60, a protein required for recombinational repair in fission yeast. Cds1 activation triggers Rad60 phosphorylation and nuclear delocalization regulating recombination events at stalled replication forks (95). While it is yet to be determined whether CHK2 activation might also play a role in repairing irofulven-elicited DNA lesions, it has been reported that irofulven-induced DNA damage is repaired by transcription-coupled nucleotide excision repair (TC-NER) (21). Therefore, it can be speculated that the CHK2 activation by irofulven might be the result of stalled replication machinery. Supporting this idea, CHK2 activation by irofulven in ovarian cancer cell lines has been found to be replication and transcription-dependent (data not shown). Interestingly, it has also been reported that DNA double-stranded breaks (DSB) are observed in irofulven-treated cells (96). Therefore, having a better understanding of the types of lesions formed by irofulven recognition and signaling pathway evoked by this drug.

In response to IR-induced DNA damage, ATM or ATR directly phosphorylates p53, NBS1, SMC1, MDM2, and BRCA1 (23, 30–37, 50–53). Activation of NBS1, SMC1, and BRCA1 plays a very important role in S and G2/M checkpoint control (22, 23, 30–41, 50–53, 66, 97, 98). Activation of SMC1 has also been shown to contribute to radiosensitivity (33). Recent studies demonstrated that parallel pathways exist in radiation-induced intra-S phase checkpoint. ATM phosphorylates CHK2 and NBS1, two branches of the ATM-mediated DNA damage response pathway (ATM-CHK2-CDCA5A-CDK2 and ATM-NBS1-MRE11-RAD50), each of which partially controls intra-S phase arrest (30, 32, 34, 65, 66, 99). We demonstrated in this study that irofulven induces phosphorylation of NBS1 on Ser343, SMC1 on Ser957, and p53 on Ser383 in an ATM-dependent manner, and induces p53 phosphorylation on Ser383-dependent on CHK2 status. The potential roles of NBS1, SMC1, and BRCA1 in irofulven-induced DNA damage response, CHK2 activation, and chemosensitivity provide avenues for future research.

It has been previously determined that CHK2 regulates G1 arrest by activating p53 (54–56), S phase checkpoint by phosphorylating CDC25A (65–67), or G2/M transition by phosphorylating CDC25C (42, 73, 74) following DNA damage. However, controversy exists regarding the role of CHK2 in cell cycle regulation. One report suggested that CHK2 is dispensable for p53-mediated G1 arrest (77), while other studies demonstrated that cells from CHK2 knockout mice have normal S phase and G2/M transition (56, 57). Additionally, it has been argued that it is unlikely CHK1 and CHK2 are regulators of p53 or the G1 and G2 checkpoints activated by IR (78, 79). Yet, CHK2 has been shown to play a partial role in controlling the S phase checkpoint upon IR treatment (66). In this study, we found that CHK2 contributes to the S phase arrest induced by irofulven. By BrdU labeling and analysis of BrdU-positive cells, S phase delay between 9- and 12-h period in irofulven-treated, BrdU-positive CHK2−/− and CHK2+/+ cells was observed. An increase in S phase cells was also observed in CHK2−/− cells treated with irofulven, but the number of cells arrested in S phase in CHK2+/+ cells was constantly higher than in CHK2−/− cells. The magnitude of differences in S phase arrest between irofulven-treated CHK2+/+ and CHK2−/− cells declined over the time. Because cells were treated with relatively low (1 × IC50) drug concentrations, it is possible that this is related to the ability of viable cells to recover from drug insult. In addition, by transfecting kinase-dead CHK2 into ovarian cancer cells, FACS analysis results indicated that S phase arrest induced by irofulven was inhibited.

CHK2 has also been implicated in apoptosis induction (56, 77, 85, 100–102). We have previously found that irofulven strongly induces JNK and ERK activation and caspase-mediated apoptosis in pancreatic cancer cell lines (19, 20). There have been reports indicating that the JNK or ERK activation in response to DNA damage might be regulated by ATM- or ATR-initiated pathways (103–107). Therefore, the possible role of CHK2 activation in irofulven-induced cell death and the link between DNA-damage response and MAPK activation induced by irofulven remain to be elucidated.

In summary, irofulven, a novel anticancer agent, activates a DNA damage signaling pathway by triggering ATM-dependent activation of NBS1, SMC1, CHK2 and p53 activation. The degree of CHK2 activation is dependent on both irofulven concentration and length of exposure. Furthermore, it was demonstrated that CHK2 activation contributes to irofulven-induced S phase arrest.

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