Regulation of FANCD2 by the mTOR Pathway Contributes to the Resistance of Cancer Cells to DNA Double-Strand Breaks

Changxian Shen1, Duane Oswald1, Doris Phelps1, Hakan Cam1, Christopher E. Pelloski2, Qishen Pang3, and Peter J. Houghton1

Abstract

Deregulation of the mTOR pathway is closely associated with tumorigenesis. Accordingly, mTOR inhibitors such as rapamycin and mTOR-selective kinase inhibitors have been tested as cancer therapeutic agents. Inhibition of mTOR results in sensitization to DNA-damaging agents; however, the molecular mechanism is not well understood. We found that an mTOR-selective kinase inhibitor, AZD8055, significantly enhanced sensitivity of a pediatric rhabdomyosarcoma xenograft to radiotherapy and sensitized rhabdomyosarcoma cells to DNA interstrand cross-linker (ICL) melphalan. Sensitization correlated with drug-induced downregulation of a key component of the Fanconi anemia pathway, FANCD2 through mTOR regulation of FANCD2 gene transcripts via mTORC1-S6K1. Importantly, we show that FANCD2 is required for the proper activation of ATM-Chk2 checkpoint in response to ICL and that mTOR signaling promotes ICL-induced ATM-Chk2 checkpoint activation by sustaining FANCD2. In FANCD2-deficient lymphoblasts, FANCD2 is essential to suppress endogenous and induced DNA damage, and FANCD2-deficient cells showed impaired ATM-Chk2 and ATR-Chk1 activation, which was rescued by reintroduction of wild-type FANCD2. Pharmacologic inhibition of PI3K–mTOR–AKT pathway in Rh30 rhabdomyosarcoma cells attenuated ICL-induced activation of ATM, accompanied with the decrease of FANCD2. These data suggest that the mTOR pathway may promote the repair of DNA double-strand breaks by sustaining FANCD2 and provide a novel mechanism of how the Fanconi anemia pathway modulates DNA damage response and repair. Cancer Res; 73(11); 1–9. ©2013 AACR.

Introduction

Recent data have shown that cyclin-dependent kinases (CDK) are required for the processing of damaged DNA ends and checkpoint activation (1–3), but the molecular mechanism by which CDKs act is unknown. PI3K–AKT and RAS–MAPK are the predominant growth-promoting signaling pathways, which enhance CDK activity and increase cell-cycle progression. Cancer genome projects showed that PI3K–AKT and RAS–MAPK are among the most frequently mutated signaling pathways in cancers (4), and both pathways converge on mTOR signaling (5). mTOR lies at the hub of intracellular and extracellular signal transduction networks via integrating and processing multiple signals, and dictates the rates of macromolecule synthesis and hence cell growth, proliferation, and survival (6–8). Aberrant activity of mTOR signaling has been found in most cancers, making mTOR an important target for cancer drug development (7, 9, 10). We previously found that the TOR pathway promotes cell survival at the cost of elevated mutation rate in response to DNA replication stress and DNA damage in yeast (11). Data also show that inhibiting the mTOR pathway sensitizes many cancer cells to chemotherapy and radiotherapy; however, the molecular mechanism by which this occurs is largely unclear (12–14).

These observations suggest that mTOR signaling maintains some components of the DNA damage response (DDR) and repair response. Besides homologous recombination, most if not all DNA repair systems are error prone and the major function of DNA damage checkpoint and repair systems is to maximize cell survival. It was intriguing, therefore, to test whether mTOR signaling modulates DDR and enhances DNA damage repair in cancer cells.

In this study, using pediatric rhabdomyosarcoma models in vitro and in vivo, we have investigated the functional linkage between the mTOR pathway and DDR, and found that mTOR signaling sustains FANCD2-mediated signaling pathways to promote DDR via potentiating the ATM checkpoint activation in response DNA double-strand breaks (DSB).

Materials and Methods

Drugs

AZD8055, MK2206, and PD0332991 were provided by AstraZeneca, Merck, and Pfizer, respectively. Rapamycin was from

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the National Cancer Institute drug repository (Frederick, MD). Melphalan, cisplatin, mitomycin C, cycloheximide, and hydroxyurea were purchased from Sigma.

**Solid tumor xenografts studies**
CB17SC scid CD1 female mice (Taconic Farms) were used to propagate subcutaneously implanted rhabdomyosarcoma. All mice were maintained under barrier conditions and experiments were carried out using protocols and conditions approved by the Institutional Animal Care and Use Committee. AZD8055 was administered per os daily at 20 mg/kg/day. Rapamycin was dissolved in dimethyl sulfoxide (5% final concentration) and diluted in 5% Tween-80 in water and administered intraperitoneally daily at a dose of 5 mg/kg. Tumors were harvested after treatment on day 1 or 4 at different timepoints.

**Cells, siRNAs, and plasmids**
Rhabdomyosarcoma Rh30 cells were cultured in RPMI-1640 (GIBCO) supplemented with 10% heat-inactivated FBS (GIBCO). Control and ON-TARGETplus SMARTpool siRNAs of CDK4, CDK6, mTOR, and FANCD2 were purchased from Dharmacon. Plasmid pcdNA3 Myr-AKT1 was a gift of William S. Sellers (Novartis, Cambridge MA; Addgene plasmid 9008). pcdDNA-DEST40 and pcdDNA-DEST40-FANCD2 plasmids were provided by Niall G. Howlett (Moffitt Cancer Center). S6K1 knockout mouse embryonic fibroblast (MEF) was provided by George Thomas (University of Cincinnati, Cincinnati, OH). MEF cells were cultured in Dulbecco's Modified Eagle Medium (GIBCO) supplemented with 10% heat-inactivated FBS (GIBCO). Lipofectamine 2000 was from Invitrogen and transfection of siRNA or plasmids in Rh30 cells was conducted according to the manufacturer's instructions.

**Immunoblotting and immunofluorescence staining**
Cells were lysed on ice in radioimmunoprecipitation assay buffer (Cell Signaling Technology) supplemented with protease inhibitors and phosphatase inhibitor (Roche), and 1 mM phenylmethylsulfonyl fluoride (Sigma). Immunoblots were probed with the following antibodies: S6, pS6 (S235/236), AKT, pAKT (S473), pGSK3β, pGSK3β (S21), pGSK3β (G20), mTOR, pS6K1 (S235/236), 4E-BP1, p4E-BP1 (T37/46), β-actin, Myc, GAPDH, Rb, pRb (S780); Cell Signaling Technology'; pH2AX (S139; Upstate); PARP1, cleaved PARP1 (Abnova); FANCD2 (Epitomics); FANCC, FANCA (Abcam); RAD51, CDK4, CDK6 (Santa Cruz). For immunofluorescence staining, cells grown on sterile cover slips were fixed in 100% cold methanol, washed with PBS, and blocked with 1% bovine serum albumin (BSA) in PBS. Cells were incubated with the γH2AX antibody (Clone JBW301, Upstate) diluted in 1% BSA in PBS for 60 minutes, and washed in 1 X PBS. The slides were additionally incubated with the FANCD2 antibody diluted in 1% BSA in PBS for 60 minutes and washed in 1 X PBS. Secondary antibodies Alexa Fluor 488 goat anti-mouse immunoglobulin G (IgG) and rhodamine (TRIT)-conjugated goat anti-rat IgG were from Invitrogen. Sample was simultaneously stained with 4′, 6-diamidino-2-phenylindole (DAPI) to detect nuclei. The fluorescence was viewed with a Zeiss axioskop 2 microscope equipped with differential interference contrast, epi-fluorescence, and UV-blocking filter sets. Images were acquired with a Micromax CCD camera (Princeton Instruments Inc.) and IP lab software (Scanalytics).

**RNA isolation, cDNA synthesis, and reverse transcriptase-PCR**
Total RNA from cultured cells was extracted with mirVana miRNA Isolation Kit (Ambion) according to the total RNA isolation protocol. Reverse transcription was conducted using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was conducted on the 7900HT Fast Real-Time PCR System using the TaqMan Universal Mastermix II. Human and mouse FANC2 expression was quantified in real time with FANC2-specific FAM dye-labeled MGB probes and normalized to GAPDH, MYCN, or β-actin (Applied Biosystems).

**Cell-cycle analysis and colony formation assay**
Following treatment, cells were trypsinized, fixed with 70% ethanol, and stored at 4°C for subsequent fluorescence-activated cell sorting analysis of DNA content. For colony formation assay, 500 cells were plated in a 10 cm Petri dish. Two weeks after treatment, cells were washed with PBS, fixed with cold methanol for 15 minutes under –20°C, and stained with 1% Crystal Violet (Fisher) in 25% methanol for 15 minutes. The dishes were washed with water extensively and the blue colonies were counted.

**Results**
**An mTOR kinase inhibitor sensitizes cancer cells to radiotherapy and chemotherapy**
Several mTOR-selective kinase inhibitors have recently been reported that in combination with rapalogs, abrogate mTOR complex 1 (mTORC1)-mediated phosphorylation of 4E-BP1 (15, 16). We previously showed that AZD8055 is a potent and specific mTOR kinase inhibitor (17). To test the involvement of mTOR in DDR, we examined the effect of AZD8055 on ionizing radiation using the Rh30 xenograft model. Mice were irradiated (2 Gy/tumor day) using whole body shielding to a range of total doses (from 10–40 Gy) with or without AZD8055 at various starting volumes. In the radiation alone group, radiation treatment induced tumor volume regressions, but tumors regrew and progressed in 14 of the 18 mice (78% failure rate) with a radiation dose per volume of tumor of 60 Gy/cm³. In contrast, only in 4 of the 15 mice did tumors regress in the combination XRT-AZD8055 treatment arm (Table 1) with dose

<table>
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<th>Group</th>
<th>Mean dose</th>
<th>Failures/total</th>
<th>Failure rate</th>
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<tr>
<td>XRT</td>
<td>60 Gy/CC</td>
<td>14/18</td>
<td>78%</td>
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<tr>
<td>XRT + AZD</td>
<td>27 Gy/CC</td>
<td>4/15</td>
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**Abbreviations:** XRT, X-ray treatment; AZD, AZD8055.
per volume of 27 Gy/cm³; a more than 50% reduction in radiation dose with a similar reduction in failure rate. In Rh30 xenografts, AZD8055 has limited single-agent activity, slowing tumor progression, but does not induce either stable disease or tumor regression (17). Thus, this mTOR kinase inhibitor may be a promising radiosensitizer.

We previously reported that rapamycin enhanced the activity of the bifunctional alkylating agent, cyclophosphamide, against several pediatric tumor models (18). To extend this observation in vitro, we checked whether AZD8055 sensitizes cultured Rh30 cells to DNA interstrand cross-linker (ICL) melphalan, which produces DNA DSB. We pretreated Rh30 cells with AZD8055 for 16 hours, and transiently exposed the cells to melphalan for 2 hours. Drugs were washed away, and survival was assayed by colony formation. AZD8055 or transient insult with melphalan resulted in 16% and 18% loss of cell viability, respectively. Pretreatment with AZD8055 followed by transient melphalan exposure led to a significant decrease in colony formation (66%; Fig. 1A and B). Thus, targeting mTOR kinase sensitizes cancer cells to ICL-based chemotherapies.

The mTOR pathway controls FANCD2

The above data suggest that mTOR signaling plays an important role for cancer cells to survive DSB-producing agents. This may be not due to the decrease of ATM, ATR and DNA-PKcs, as AZD8055 did not alter the protein levels of ATR, RAD51, and DNA-PKcs but slightly increased ATM (Fig. 1C). Of note, exposure to AZD8055 resulted in marked decrease in FANC2D, but not other components of the Fanconi anemia pathway (FANCC, FANCA). Fanconi anemia pathway-deficient cells display phosphorylation of H2AX, a marker of DNA strand breaks, and defect of DNA damage checkpoint in response to DNA damage (19, 20). Fanconi anemia cells, such as FANC2D-deficient PD20 lymphoblast cells, display high levels of γH2AX, and reexpression of FANC2D in PD20 cells reduced γH2AX levels (Fig. 1D; refs. 19, 20). Similarly, knockdown of FANC2D by siRNA led to increased γH2AX in Rh30 cells (Fig. 1E). Interestingly, AZD8055 downregulated FANC2D and induced γH2AX, whereas ectopic overexpression of FANC2D partially attenuated AZD8055-induced γH2AX (Fig. 1F). These results led us to hypothesize that mTOR signaling may regulate DDR and hence DSBR by controlling FANC2D signaling. AZD8055 treatment reduced the levels of FANC2D in a time-dependent manner (Supplementary Fig. S1A). Similarly, knockdown of mTOR by siRNA decreased FANC2D (Fig. 1G).

In contrast to melphalan that causes ICL, inhibition of mTOR signaling did not result in the monoubiquitination of FANC2D in Rh30 cells (Fig. 1H). In addition, overexpression of wild-type AKT1 (Supplementary Fig. S1B) or myristoylated AKT1 (myr-AKT1; Supplementary Fig. S1C) in Rh30 cells increased FANC2D and was associated with enhanced activity of the AKT pathway as shown by the elevation of the pGSK3β-S9.

Figure 1. The mTOR pathway promotes cell survival in response to melphalan by sustaining FANC2D. A, five hundred cells were plated in 10 cm dish for 6 hours, then AZD8055 (2 μmol/L) was applied for 16 hours. Melphalan (0.2 μg/mL) was added to untreated or AZD8055-treated cells for 2 hours. The drugs were washed away. Two weeks later, the colonies were counted. Shown is a representative of 3 independent experiments. B, quantification of the colonies in Fig. 1A. Error bars, mean ± SD (n = 3). C, Rh30 cells were treated with AZD8055 (2 μmol/L) for 16 hours. Total proteins were extracted for immunoblotting. D, lymphoblast PD20 cells derived from a patient with FANC2D were stably transfected with empty vector (pMMP-Puro) or wild-type FANC2D plasmid (pMMP-wt-FANC2D). FANC2D and γH2AX were detected by immunoblotting. –, pMMP-Puro; +, pMMP-wt-FANC2D. E, Rh30 cells were transfected with control or FANC2D siRNA. Forty-eight hours later, total proteins were extracted for immunoblotting. F, Rh30 cells were transfected with vector or FANC2D plasmid. Twenty-four hours later, AZD8055 (2 μmol/L) was added for additional 24 hours. Total proteins were extracted for immunoblotting. G, Rh30 cells were transfected with control or mTOR siRNA. Forty-eight hours later, total proteins were extracted for immunoblotting. H, Rh30 cells were treated with AZD8055 (AZD, 2 μmol/L) or melphalan (MP, 2 μg/mL) for the time indicated. Total proteins were extracted for immunoblotting. UT, untreated. Upper band of FANC2D staining showed the monoubiquitination of FANC2D. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin served as loading controls.
signal. These data showed that AKT–mTOR signaling controls the levels of FANCD2. To test this further, we treated cultured rhabdomyosarcoma Rh30 cells with mTORC1-specific inhibitor rapamycin, AZD8055 and AKT kinase inhibitor MK2206 (21). Rapamycin inhibited the phosphorylation of pS6K1 (T389), a marker of mTORC1 activity, but increased the phosphorylation of AKT at S473 due to the mTORC1-S6K1-IRS–negative feedback loop (22). Treatment with either AZD8055 or MK2206 resulted in absence of the phosphorylation of both pS6K1 (T389) and pAKT (S473), indicating inhibition of both mTORC1 and mTORC2. Similar to AZD8055, MK2006 reduced FANCD2 although the effect of rapamycin was less pronounced (Supplementary Fig. S1D), showing that AKT–mTOR pathway controls FANCD2.

Our data suggest that mTOR signaling may be involved in the DDR and repair of DSB by regulating the Fanconi anemia signaling pathway. To test this in vivo, we next checked FANCD2 protein levels of the mouse xenografts of Rh18, Rh30, and Rh10 following treatment with AZD8055. Mice received AZD8055 daily for 4 days, and tumors were harvested 1, 4, 8, and 24 hours after the final dose. AZD8055 efficiently and rapidly inhibited both the mTORC1 and mTORC2, as shown by the decrease of p4E-BP1-T37/46, pS6-S235/6, and pAKT-S473 signals 1 hour after treatment (Fig. 2A and B). At 24 hours after AZD8055 dosing, FANCD2 was markedly reduced in all of the 3 tumor models (Fig. 2C). As AZD8055 inhibits both mTORC1 and mTORC2, we additionally checked the FANCD2 of the mouse tumor xenografts Rh30 and Rh18 treated with rapamycin. Similar to AZD8055, inhibition of mTORC1 by rapamycin resulted in apparent downregulation of FANCD2 in vivo (Fig. 2D). These in vivo and in vitro data suggest that FANCD2 is regulated by the mTORC1 pathway.

mTORC1-S6K1 signaling controls transcription of FANCD2

The above results showed that inhibition of mTORC1 led to decreased FANCD2 both in tumor xenografts and cultured cells. As TOR signaling has been implicated in both translation and transcription, we probed the mechanism by which this pathway regulates FANCD2 levels. We determined the mRNA levels of FANCD2 following inhibition of mTOR signaling by real-time reverse transcriptase PCR (RT-PCR). Treatment of Rh30 cells with AZD8055 resulted in a progressive decrease in FANCD2 mRNA (Fig. 3A). Similarly, rapamycin and MK2206 also decreased FANCD2 mRNA (Fig. 3B). Conversely, overexpression of AKT1 increased the mRNA of FANCD2 (Fig. 3C). Thus mTOR signaling seems to regulate either transcription or transcript stability of FANCD2 gene.

From yeast to mammalian cells, the TOR pathway promotes cell-cycle progression by increasing the activity of CDKs (8). Indeed, AZD8055 robustly reduced both total and pRb-S780 (Fig. 3D). Recently, an essential role for CDKs in processing of damaged DNA ends and checkpoint activation has been described (1). Our data show that mTOR coordinates controls the proteins and mRNAs of FANCD2. These observations suggest that mTOR controls the gene expression of FANCD2 by regulating CDKs. To further test this observation, we treated Rh30 cells with rapamycin, AZD8055 or MK2206, in parallel with a CDK4/6-specific inhibitor PD0332991 (23, 24), and determined FANCD2 by immunoblotting. Similar to AZD8055 and MK2206, PD0332991 significantly reduced FANCD2 (Fig. 3E). We further analyzed the FANCD2 mRNAs of Rh30 cells treated with PD0332991. Consistent with the decreased protein levels, PD0332991 reduced FANCD2 mRNAs (Fig. 3F), showing that the gene transcription/transcript stability of FANCD2 is indirectly controlled by CDKs. We next reduced CDK4 and CDK6 by siRNAs in Rh30 cells. Knockdown of CDK4 led to reduction of both FANCD2 protein (Supplementary Fig. S2A) and mRNA (Supplementary Fig. S2B). Interestingly, downregulation of CDK6 by siRNA increased both the protein and mRNA levels of FANCD2, accompanied by an enhanced pRb-S780 signal (Supplementary Fig. S2A). These data showed that FANCD2 is positively controlled by CDK4, which is consistent with the finding that FANCD2 is regulated by Rb-E2F1 (25).

We further tested whether FANCD2 mRNA is controlled by mTORC1 signaling, by examining whether S6K1 knock out MEF cells (S6K1/−/− MEF). S6K1/−/− MEFs showed significant downregulation of FANCD2 protein (Fig. 3G) and mRNA (Fig. 3H), and rapamycin did not further downregulate FANCD2 in

![Figure 2. FANCD2 is controlled by mTOR signaling in pediatric rhabdomyosarcoma in vivo. A and B, pediatric rhabdomyosarcoma Rh10 and Rh30 tumor xenograft models were propagated subcutaneously in severe combined immunodeficient mice and were treated with mTOR kinase inhibitor AZD8055 at 20 mg/kg/d. Tumors were harvested 1, 4, 8, and 24 hours after treatment on day 4 and were pulverized under liquid N2. Total proteins were extracted for immunoblotting. C, mice bearing subcutaneous rhabdomyosarcoma xenografts, Rh18, Rh10, and Rh30 tumor xenografts, were treated with mTOR kinase inhibitor AZD8055 (20 mg/kg daily). Tumors were harvested 24 hours after the fourth dose administration. Total proteins were extracted for immunoblotting to detect FANCD2. D, pediatric rhabdomyosarcoma Rh18 and Rh30 tumor xenograft models were propagated subcutaneously in severe combined immunodeficient mice and were treated with rapamycin at 5 mg/kg/d. Tumors were harvested 24 hours after treatment on day 1. Total proteins were extracted for immunoblotting. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.](image)
mTOR kinase seems to control FANCD2 principally via the mTORC1–S6K1 pathway. FANCD2 is required for the activation of both ATM-Chk2 and ATR-Chk1 checkpoints. It was recently reported that FANCD2 mediates the nucleolytic incisions near the ICL and leads to DNA DSB (26, 27), which may result in ATM-Chk2 activation and phosphorylation of H2AX. This model suggests that FANCD2 may be essential for ATM-Chk2 activation in the early steps of Fanconi anemia signaling-mediated repair of ICL-induced DNA lesions. To test this hypothesis, we first compared the status of ATR-Chk1 and ATM-Chk2 activation in response to hydroxyurea in PD20 cells and the PD20 cells reintroduced with wild-type FANCD2 by immunoblotting. Hydroxyurea-treated PD20 cells displayed decreased pChk1-S345, pATM-S1981, and pChk2-T68 signals compared with those of PD20 with FANCD2 reintroduced (Fig. 4B). We additionally tested...
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During the early response to ICL, FANCD2 is required for the induction by melphalan (Fig. 5D). These results showed that and FANCD2 siRNA reduced the phosphorylation of H2AX plus melphalan or AZD8055 plus melphalan. Both AZD8055 and FANCD2 siRNA reduced the phosphorylation of H2AX by melphalan (Fig. 5D). These results showed that during the early response to ICL, FANCD2 is required for the proper phosphorylation of H2AX and hence activation of ATM, but not essential for ATR-Chk1 activation, providing evidence for the proposed model of the function of FANCD2 in response to ICL (26, 27).

Our observations, that FANCD2 is required for the timely ATM-Chk2 checkpoint activation in response to ICL and FANCD2 is controlled by mTOR signaling, suggest that the mTOR pathway is essential for the timely activation of ATM-Chk2 checkpoint in response to ICL. To test this, we treated PD20 cells with AZD8055 for 16 hours, followed by addition of melphalan for 5 hours. Although FANCD2-deficient PD20 cells displayed impaired Chk2 activation in response to melphalan (Fig. 4C), PD20 cells rescued by wild-type FANCD2 showed robust Chk2 activation, which was abolished by AZD8055 accompanied with reduction of FANCD2 protein (Fig. 6A). Similarly, in Rh30 cells, melphalan-induced pATM-S1981 signals were reduced by either AZD8055 or MK2206, accompanied with downregulation of FANCD2 (Fig. 6B). In addition to melphalan, AZD8055 also abolished the activation of Chk2 by mitomycin C (Fig. 6C). This reduction seems independent of the proteasome pathway because MG132 did not affect AZD8055-induced reduction of FANCD2 and melphalan-mediated Chk2 activation (Fig. 6C). Thus, these data support the hypothesis that mTOR pathway is required for an early step(s) in ATM-Chk2 checkpoint activation in response to ICL.

Discussion

In this study, we observed that an mTOR-selective kinase inhibitor sensitized pediatric rhabdomyosarcoma xenografts to radiotherapy and rhabdomyosarcoma cells to ICL. Control of FANCD2 transcripts and protein levels by mTOR signaling (Supplementary Fig. S3A) may, at least partially, contribute to the sensitization of cancer cells. Furthermore, FANCD2 is required for timely ATM-Chk2 checkpoint activation in response to ICL in Rh30 cells, suggesting that one of the mechanisms that Fanconi anemia signaling promotes cell survival of ICL is through potentiation of ATM-Chk2 activation (Supplementary Fig. S3B).

Importantly, therapy-using agents that damage DNA and radiotherapy are essential components of many clinical protocols, and form the backbone for curative treatment of several childhood malignancies. ICL-based chemotherapies, such as cisplatin or cyclophosphamide, are first-line drugs for treatment of many cancers. Our observation that cancer cells are sensitized to melphalan and ionizing radiation by an mTOR kinase inhibitor provides a strategy for cancer therapy by combination of targeting mTOR signaling with these DNA-damaging modalities.

Our findings may provide the explanation for recent reports about the involvement of mTOR signaling in DDR. Several groups found that inhibition of mTOR signaling resulted in γH2AX and impairment of DDR following exposure to DNA-damaging agents in cultured cells (31–33). The key function of DDR is to maximize cell survival by promoting DNA repair (30). Induction of γH2AX following inhibition of mTOR signaling suggests impairment of DNA strand break repair. Moreover, inhibition of mTOR signaling in MCF-7 cells was shown to result in impaired recruitment of BRCA1 and RAD51 to DNA.
repair foci (34). BRCA1 and RAD51 promote repair of DNA DSB by homologous recombination (HR). Fanconi anemia signaling is essential for cells to survive DNA ICL by coordinating DNA damage repair through translesion DNA synthesis (TLS), nucleotide excision repair (NER), and HR (35). Therefore, downregulation of FANCD2 by inhibition of mTOR signaling may, at least partially, explain the impaired DDR in response to DNA-damaging agents.

Recently, it was shown that ATM is synthetically lethal with Fanconi anemia (28). Fifteen FANC genes have been identified in Fanconi anemia or Fanconi anemia-like patients (35, 36). FANCD2 is the key component of Fanconi anemia signaling. Activation of Fanconi anemia pathway by ICL leads to mono-ubiquitination of FANCI-FANCD2 by Fanconi anemia core E3 ubiquitin ligase complex. Activated FANCI-FANCD2 is recruited to DNA damage sites, and helps endonucleases to cut both sides of ICL to generate DNA strand breaks, and promotes TLS, NER, and Rad51-mediated HR together with BRCA1 and BRCA2 (FANC1; ref. 37). FANCD2-mediated nucleolytic incisions near the ICL result in a DNA DSB (26, 27), predicted to activate ATM-Chk2. In support of this model, our data show that FANCD2 is required for ATM-Chk2 activation in the early steps of Fanconi anemia signaling-mediated repair of ICL-induced DNA lesions.

Our observation that FANCD2 is controlled by mTOR signaling and mTOR inhibition led to attenuation of ATM-Chk2 checkpoint activation by ICL provides the molecular mechanism by which inhibition of mTOR signaling sensitizes cancer cells to radiotherapy and chemotherapy. FANCD2 is required for repair of DSB and intra-S-phase checkpoint activation (20, 35). Although the Fanconi anemia pathway is essential for cells to survive ICL, it can be activated by most DNA damage including DSB, single-strand breaks, base damage, intra- and interstrand DNA crosslinks, and DNA replication block (37). We found attenuated activation of ATR-Chk1 and ATM-Chk2 in PD20 cells under normal growth conditions.
hydroxyurea-induced DNA replication stress, and melphalan-mediated DNA damage. ATR-Chk1 and ATM-Chk2 checkpoints are the central genome surveillance systems to maintain cell survival in response to endogenous and exogenous DNA damage. DNA damage or replication stress leads to ATR-Chk1 and ATM-Chk2 checkpoint activation in response to DNA ICL-induced DNA damage. A, lymphoblast PD20 cells stably transfected with pMMP-Puro or pMMP-wt-FANCD2 were treated with AZD8055 (2 μmol/L) for 16 hours, then melphalan (MP, 2 μg/mL) was added alone or in combination with AZD8055 and incubated for 6 hours. Immunoblotting was done to detect FANCD2, Chk2, and pChk2-T68. –, pMMP-puro; +, pMMP-wt-FANCD2. B, Rh30 cells were treated with rapamycin (100 ng/mL), AZD8055 (2 μmol/L), or MK2206 (10 μmol/L) for 16 hours. Then melphalan (MP, 2 μg/mL) was added alone or in combination as indicated and incubated for 6 hours. Immunoblotting was done to detect FANCD2 and pATM-S1981. C, Rh30 cells were treated with rapamycin (100 ng/mL), AZD8055 (2 μmol/L), or MK2206 (10 μmol/L) for 16 hours. Then melphalan (MP, 2 μg/mL), cisplatin (5 μmol/L), and mitomycin C (2 μmol/L) were added alone or in combination as indicated for 6 hours. Two hours before protein extraction, MG132 (2 μmol/L) was added as indicated. FANCD2 and pChk2-T68 were detected by immunoblotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as loading control.

Figure 6. The mTOR pathway is required for ATM-Chk2 checkpoint activation in response to DNA ICL-induced DNA damage. A, lymphoblast PD20 cells stably transfected with pMMP-Puro or pMMP-wt-FANCD2 were treated with AZD8055 (2 μmol/L) for 16 hours, then melphalan (MP, 2 μg/mL) was added alone or in combination with AZD8055 and incubated for 6 hours. Immunoblotting was done to detect FANCD2, Chk2, and pChk2-T68. –, pMMP-puro; +, pMMP-wt-FANCD2. B, Rh30 cells were treated with rapamycin (100 ng/mL), AZD8055 (2 μmol/L), or MK2206 (10 μmol/L) for 16 hours. Then melphalan (MP, 2 μg/mL) was added alone or in combination as indicated and incubated for 6 hours. Immunoblotting was done to detect FANCD2 and pATM-S1981. C, Rh30 cells were treated with rapamycin (100 ng/mL), AZD8055 (2 μmol/L), or MK2206 (10 μmol/L) for 16 hours. Then melphalan (MP, 2 μg/mL), cisplatin (5 μmol/L), and mitomycin C (2 μmol/L) were added alone or in combination as indicated for 6 hours. Two hours before protein extraction, MG132 (2 μmol/L) was added as indicated. FANCD2 and pChk2-T68 were detected by immunoblotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as loading control.

References


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