Invest New Drugs (2011) 29:514–522
DOI 10.1007/s10637-009-9361-2

SHORT REPORT

A novel Chk inhibitor, XL-844, increases human cancer cell radiosensitivity through promotion of mitotic catastrophe

Oliver Riesterer · Fumihiko Matsumoto · Li Wang · Jessica Pickett · David Molkentine · Uma Giri · Luka Milas · Uma Raju

Received: 21 October 2009 / Accepted: 20 November 2009 / Published online: 22 December 2009

Summary Check point kinases (Chk) play a major role in facilitating DNA repair upon radiation exposure. We tested the potency of a novel inhibitor of Chk1 and Chk2, XL-844 (provided by Exelixis Inc., CA, USA), to radiosensitize human cancer cells grown in culture and investigated the underlying mechanisms. HT-29 cells (a human colon cancer line) were exposed to XL-844, radiation, or both, and assessed for clonogenic cell survival. Treatment-dependent effects on phosphorylated forms of Chk proteins were assessed by Western blots. Further mechanistic investigations in HT-29 cells included cell cycle analysis by flowcytometry and assessment of DNA repair kinetics by immuno-cytochemistry (ICC) for nuclear appearance of the phosphorylated form of histone 2AX protein (γ-H2AX) staining. Cells undergoing mitotic catastrophe were identified by irregular pattern of mitotic spindle markers α and γ-tubulin staining by ICC. XL-844 enhanced radiosensitivity in a dose and schedule-dependent manner and the enhancement factor was 1.42 at 0.5 survival fraction. Mechanistically XL-844 abrogated radiation-induced Chk2 phosphorylation, induced pan-nuclear γ-H2AX, and prolonged the presence of radiation-induced γ-H2AX foci, and promoted mitotic catastrophe. In conclusion, our data showed that inhibition of Chk2 activity by XL-844 enhanced cancer cell radiosensitivity that was associated with inhibition of DNA repair and induction of mitotic catastrophe.

Keywords Radiosensitivity · Inhibitor of Check point kinases · XL-844 · Mitotic catastrophe · Pan-nuclear γ-H2AX

O. Riesterer · F. Matsumoto · L. Wang · J. Pickett · D. Molkentine · U. Giri · L. Milas · U. Raju (✉)
Department of Experimental Radiation Oncology,
The University of Texas M. D. Anderson Cancer Center,
Unit 66, 1515 Holcombe Blvd.,
Houston, TX 77030, USA
e-mail: uraju@mdanderson.org

Present Address:
O. Riesterer
Department of Radiation Oncology, University Hospital Zurich,
Ramistrasse 100,
8091 Zurich, Switzerland

Introduction

Check point kinases 1 and 2 (Chk1 and Chk2) play a major role in mediating the signal transduction pathways that are activated upon DNA damage induced by cytotoxic agents such as radiation [1]. Radiation-induced cascade of molecular events are attributed to the activation of ataxia telangiectasia, mutated (ATM) or ATM and Rad3-related (ATR) proteins [2–6], which thus lead to the phosphorylation of Chk proteins [7–9]. Chk1 and/or 2 activation leads to cdc-2 (Cdk1) inhibition through inactivation of cdc25 by phosphorylation thereby causing G2-arrest [10]. Cdc-2 is a cyclin-dependent kinase (CDK) which, when bound to cyclin B, allows a dividing cell to enter into mitosis from G2 cell cycle phase.

Radiation-induced cell cycle arrest in the G2 phase enables the cells to undertake DNA repair processes before entering into mitosis to ensure genomic stability resulting in increased cell survival, thus antagonizing the effects of radiation [11, 12]. Premature entry into mitosis may result in mitotic catastrophe, associated with the formation of large cells, lobulated micronuclei, multi- or unipolar spindles, and/or lagging chromosomal material and subsequently in cell death. The inhibition of Chk proteins in cancer cells is therefore a rational approach to sensitizing these cells to radiation-induced cell killing. XL-844 (formerly, EXEL-9844) is a novel agent that selectively

inhibits the kinase activity of Chk proteins [13]. As a single agent, XL-844 exhibited moderate antitumor activity in pancreatic cancer cells; however, when combined with gemcitabine, a chemotherapeutic agent, XL-844 displayed a synergistic effect on clonogenic cell survival and substantially enhanced gemcitabine-induced cell killing [13]. In addition, XL-844 increased the gemcitabine-induced DNA damage, blocked cdc25A phosphorylation, abrogated the gemcitabine-induced S phase checkpoint and induced premature mitotic entry [13].

However, no studies of the effect of XL-844 given with radiation therapy have been performed so far. A report on a broad-spectrum serine-threonine kinase inhibitor 7-hydroxy-staurosporine, also known as UCN-01, showed a synergistic interaction with radiation that was associated with inhibition of Chk activity [14]. A recent report showed that inhibition of Chk-2 using PV1019 potentiated the effects of camptothecins and radiation [15]. Recently, XL-844 entered into phase I clinical trials along with two other Chk inhibitors, AZD7762 and PF00477736 [16]. The present study was undertaken to determine the efficacy of XL-844 in enhancing the sensitivity of human HT-29 colon carcinoma cells to radiation and also to investigate the underlying cellular mechanisms of this sensitization.

Materials and methods

Cell culture

Human cancer cell line, HT-29 (colorectal adenocarcinoma) was obtained from American Type Culture Collection, (Manassas, VA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium supplemented with fetal calf serum (10%) and penicillin-streptomycin (10,000 U/mL).

XL-844

XL-844 was obtained from Exelixis Inc. (CA, USA) as a 10 mM solution dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C. Dilutions of XL-844 for the treatment of cells were made using DMSO and culture media.

Clonogenic cell survival assays

Cells in culture were exposed to XL-844 (500 nM or 1,500 nM) for 16 h, radiation treatment (γ-rays using a ^{137}Cs source, 3 Gy/min) of 2 Gy, 4 Gy, or 6 Gy, or a combination of both. Three different schedules were tested. (1): Cells were pretreated with 500 nM or 1,500 nM of XL-844 for 16 h followed by removal of the drug and then radiation exposure. (2): Cells were exposed to radiation first and immediately after radiation, XL-844 was added to the cells and incubated for 16 h. (3): Cells were exposed to radiation and 24 h later, cells were treated with XL-844 for 16 h. After treatments, cells were assayed for colony-forming ability by trypsinizing and replating them in specified numbers in 100-mm dishes in drug-free medium. After 10–12 days of incubation, the cells were stained with 0.5% crystal violet in absolute ethanol, and colonies with more than 50 cells were counted under a dissection microscope. Radiation survival curves were plotted after normalizing for the cytotoxicity induced by XL-844 alone. Clonogenic survival curves were constructed from at least two or three independent experiments by fitting the average survival levels using least-squares regression by the linear quadratic model [17].

Western blot analysis

Cells were exposed to γ-radiation followed by XL-844 treatment. Cells were collected 30 min after treatment. Whole cell lysates were subjected to Western blot analysis. The immunoreaction was visualized using an ECL-Plus detection system (Amersham, Arlington Heights, IL, USA) and analyzed using a Typhoon 9400 Scanner (Molecular Dynamics, Sunnyvale, CA, USA).

Apoptosis and cell-cycle analysis

Cells were plated in 100-mm dishes and treated with XL-844 (1,500 nM), γ-radiation, or both, where the cells were exposed to radiation and immediately after irradiation XL-844 was added to the culture media. Cells were subjected to terminal deoxy-nucleotidyltransferase (TdT) dUTP nick-end labeling (TUNEL) assay 16 h after treatment. An Apo-Direct kit (Pharmingen, San Diego, CA, USA) was used following the manufacturer's protocol, including that for staining with propidium iodide for cell-cycle analysis. Cells were analyzed by flow cytometry to quantify the numbers of apoptotic cells and the distribution of cells throughout the cell cycle.

Immuno-cytochemical analysis

Cells were grown on cover slips placed in 35-mm dishes for 48 h and then treated with XL-844, radiation, or both. After an incubation period of 30 min, 4 h or 16 h, the cells were fixed in 1% paraformaldehyde first and then in 70% ethanol and processed for immuno-fluorescent staining. Then the cells were exposed to 1% Igepal. After washing with phosphate buffered saline (PBS), cells were exposed to 5% bovine serum albumin (BSA) in PBS before the primary antibody incubation. The primary antibodies (1:300 dilution) used were rabbit anti-phosphorylated histone H2AX

(γ-H2AX) (Trevigen Inc., Gaithersburg, MD), mouse anti-α-tubulin (B7) (Santa Cruz) and mouse anti-γ-tubulin (clone GTU-88) (Sigma-Aldrich, St. Louis, MO). After overnight incubation in primary antibodies the cells were washed with PBS and then exposed to the appropriate secondary antibodies. The secondary antibodies (1:500 dilution) used were fluorescein isothiocyanate (FITC) conjugated donkey anti-rabbit immunoglobulin (IgG) (Jackson ImmunoResearch Labs., West Grove, PA, USA) for γ-H2AX and Texas Red anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) for both α-tubulin and γ-tubulin.

Statistical analysis

Statistical analysis was conducted using Student's *t* test. Group differences resulting in *p* values of less than or equal to 0.05 were considered to be statistically significant.

Results

XL-844 enhanced the sensitivity of HT-29 cells to radiation

To test the efficacy of XL-844 in enhancing cell radiosensitivity, cells were exposed to XL-844, radiation, or both. As a single agent XL-844 did not induce measurable amount of cell killing. However, it suppressed clonogenicity of these cells that was apparent in the reduced plating efficiency (PE) of XL-844 treated cells in clonogenic cell survival assay. When XL-844 was applied to these cells for 16 h, the PE of HT-29 cells was reduced to 93.7% (when a dose of 500 nM was used) and 90.7% (at a dose of 1,500 nM) of that seen in untreated controls. When combined with radiation, XL-844 elicited its radiosensitizing effect in a schedule-specific manner. To determine the optimal time of application of XL-844 in conjunction with radiation exposure, and also to determine the mode of interaction of Chk and radiation pathways, three different experimental schedules were applied: XL-844 given prior to, immediately after, or 24 h after irradiation. Radiation alone caused a dose-dependent reduction in cell survival. As far as dosing was concerned, 500 nM of XL-844 was not effective in enhancing the radiosensitivity of HT-29 cells (data not shown), however, 1,500 nM of XL-844 was effective in enhancing the radiosensitivity, which was schedule-dependent. When XL-844 was administered for 16 h before or 24 h after irradiation, no enhancement in the cellular radiosensitivity was observed (data not shown), but, when XL-844 was applied immediately after radiation exposure and left in the culture media for the next 16 h, HT-29 cells exhibited enhanced radiosensitivity (Fig. 1). The curves representing the effect of XL-844 plus radiation were normalized, *i.e.*, the colony number after drug alone

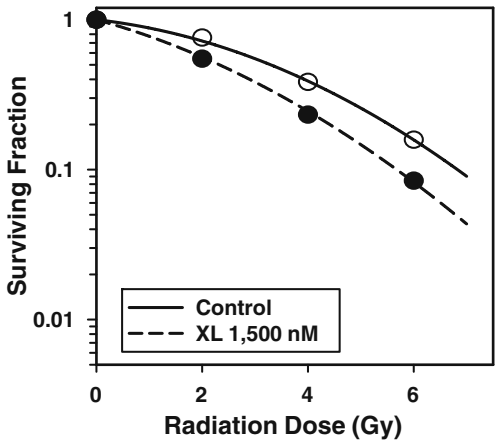

Fig. 1 Effect of XL-844 on radiosensitivity of HT-29 cells in culture. Exponentially growing cells were exposed to radiation first and immediately after irradiation, they were treated with XL-844 (1,500 nM) for 16 h. Then, cells were trypsinized and replated in specific numbers for colony formation in drug free media. Twelve days later colonies were stained, counted and survival curves were constructed with normalized values for the cytotoxicity induced of XL-844 alone. Values shown are the means ± SE from three independent experiments

was used as 1 (100% survival). The enhancement factor was calculated at the surviving fraction of 0.5 by dividing radiation dose of the control curve with that of the corresponding XL-844 plus radiation curves. The enhancement factor was 1.42. Treatment with XL-844 (1,500 nM) modified the shape of the cell survival curve by reducing the "shoulder" region on the survival curve.

XL-844 inhibited the Chk2 signaling pathway

To determine the effect of XL-844 and radiation on Chk kinases, cells were exposed to XL-844 (1,500 nM), 4 Gy of radiation, or both (XL-844 was added to the cells immediately after irradiation). Cells were collected 30 min after treatments and whole cell lysates were subjected to Western blot analysis. Radiation-induced Chk2 phosphorylation (p-Chk2) involving threonine 68 (Thr-68), was almost completely inhibited by XL-844 (Fig. 2). Interestingly, analysis of Chk1 phosphorylation (p-Chk1) involving serine 345 (Ser-345) showed that XL-844 alone and in combination with radiation significantly increased the expression levels of p-Chk1. Analysis of the levels of phosphorylated forms of cyclin-dependent kinase-1 (p-cdk1, also known as p-cdc2) showed that the cells expressed constitutively high levels of cdc2, phosphorylated at tyrosine15 (Tyr-15) (Fig. 2). Exposure to radiation did not alter the Tyr-15 phosphorylation of cdc2 protein. However, the phosphorylation of threonine 161 (Thr-161) residue of cdc2 protein was up regulated by radiation alone and also in combination with XL-844 suggesting activation of cdc2 by both treatments.

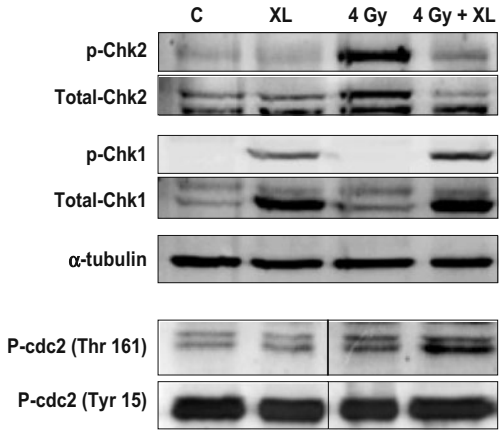

Fig. 2 Expression levels of Chk2, Chk1 and cdc2 proteins and their phosphorylated forms by Western blot analysis. Cells were exposed to 4 Gy, XL-844 (1,500 nM), or both (irradiation followed by XL-844 treatment), and collected 30 min after treatments. Cell lysates were analyzed for protein expression. Data shown are representative of three independent experiments

XL-844 suppressed the radiation-effect on cell-cycle redistribution, but no induction of apoptosis

To test whether Chk-inhibition induced apoptosis and / or altered cell-cycle distribution, cells were treated with 1,500 nM of XL-844 alone, 6 Gy of radiation alone, or the combination of both. Cells were subjected to TUNEL assay and cell-cycle analysis by flowcytometry16 h after treatment. XL-844 (1,500 μM, 16 h) as a single agent as well as in combination with 6 Gy-radiation did not induce apoptosis in these cells. However it suppressed radiation-induced alterations in cell cycle distribution. As shown in Fig. 3, radiation at a dose of 6 Gy caused cells to accumulate in the G_2 phase of the cell-cycle, increasing from a control value of 10.5% to 52%. However, combination treatment with XL-844 and radiation resulted in only 32% of cells accumulated in the G_2 phase, suggesting that XL-844 treatment suppressed partially the effect of radiation.

XL-844 induced pan-nuclear γ-H2AX and prolonged the presence of radiation-induced γ-H2AX-foci

To investigate whether the radioenhancement induced by XL-844 involved inhibition of DNA repair, HT-29 cells were subjected to γ-H2AX staining, a marker for DNA double strand breaks (DSBs). At different time points after treatment with XL-844, radiation, or both, cells were processed for immuno-cytochemical analysis. Figure 4a illustrates representative nuclei of each treatment condition at 30 min, 4 h or 16 h after the treatments. The immuno-reaction with γ-H2AX was visualized using FITC-conjugated secondary antibody. Whereas, untreated control cells exhibited no γ-H2AX foci or pan-nuclear γ-H2AX, exposure of cells to 6 Gy of radiation induced formation of γ-H2AX foci minutes after irradiation that was considerably reduced by 4 h and 16 h after irradiation (Fig. 4a, red arrow). XL-844 treatment alone did not induce any γ-H2AX foci formation, but, it generated the appearance of pan-nuclear γ-H2AX by 4 h, which was extended up to 16 h time point after treatment (Fig. 4a white arrow). Quantification of γ-H2AX foci showed that radiation exposure induced the appearance of γ-H2AX foci in >90% of nuclei and the presence of XL-844 did not have any significant effect at 0.5 h time point (Fig. 4b). By 4 h after irradiation, the number of nuclei with γ-H2AX foci had decreased to 31%. When the cells were exposed to radiation and XL-844 treatments, the number of nuclei with γ-H2AX foci plus the number of nuclei showing pan-nuclear γ-H2AX together (γ-H2AX positive) was 52% at 4 h, which was 21% more than the number generated in cells that were exposed to radiation only (Fig. 4b). Quantification of pan-nuclear appearance of γ-H2AX showed that by 4 h after XL-844 treatment, 3% of cells exhibited pan-nuclear γ-H2AX staining that was extended to 9% of cells by 16 h (Fig. 4c). By 16 h, the appearance of pan-nuclear γ-H2AX generated by XL-844, made it impossible to visualize γ-H2AX foci. Combination treatment with radiation and XL-844 had a profound effect on the formation of pan-nuclear γ-H2AX, with 25% of nuclei exhibiting pan-nuclear γ-H2AX (p=0.0002) (Fig. 4c).

XL-844 and radiation induced mitotic catastrophe

To determine whether XL-844 and radiation treatments resulted in mitotic catastrophe, co-staining with α- or γ-

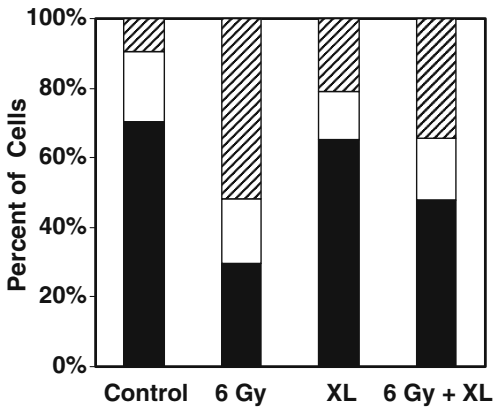

Fig. 3 Cell cycle redistribution in HT-29 cells. Cells were treated with 6 Gy of radiation, XL-844 (16 h, 1,500 nM), or both (irradiation, immediately followed by XL-844 treatment for 16 h). Then, cells were trypsinized and fixed in 70% ethanol. After washing, cells were exposed to propidium iodide/RNase solution before performing flow cytometry analysis. G1: Black; S: White; G2: Striped. Data shown are representative of two independent experiments

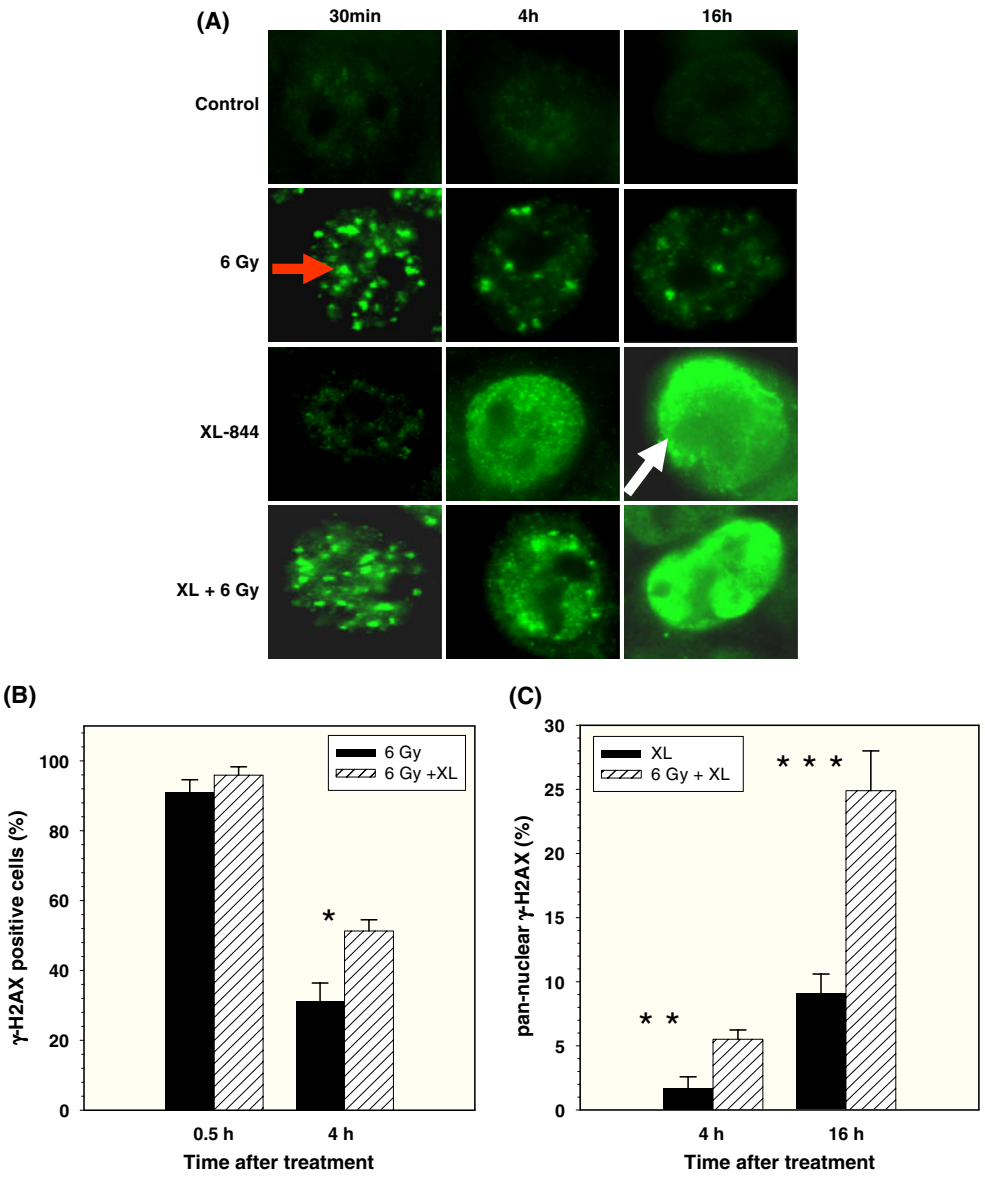

Fig. 4 Immuno-cytochemical analysis for γ-H2AX staining. **a** HT-29 cells were treated with 6 Gy, XL-844 (1,500 nM), or both (irradiation followed by XL-844 treatment). Cells were fixed 30 min, 4 h, or 16 h after treatments and stained for γ−H2AX (*green*). Shown are representative nuclei containing γ−H2AX foci (*red arrow*) and/or pan nuclear γ−H2AX (*white arrow*). **b** Quantification of γ-H2AX positive cells at 0.5 h and 4 h (including pan-nuclear γ-H2AX) after 6 Gy alone or 6 Gy + XL-844 (*p=0.007). **c** Quantification of cells displaying only pan-nuclear γ-H2AX at 4 and 16 h after treatment with XL-844 alone or both 6 Gy + XL-844. The graphs show the means ± SE of three independent experiments. For every experiment at least 200 nuclei were counted per treatment condition. **p=0.009, ***p=0.0002

tubulin (with Texas red) and γ-H2AX (with FITC, green) were performed. Further, DNA was stained with Dapi (blue). In the untreated control cell population, about 7% of cells stained positive with α-tubulin (red), showing the presence of well-defined two polar bodies with interpolar microtubules in the anaphase of mitosis showing normal anaphase components (red arrow in Fig. 5a). Whereas, the number of cells that stained positive for α-tubulin was not altered by XL-844 treatment, the staining patterns were different. Treatment with XL-844 alone or in combination with radiation induced atypical staining with α-tubulin (red) with abnormal spindle anatomy and it was associated with pan-nuclear γ-H2AX (green) staining (white arrows, Fig. 5a). In addition, staining of the DNA (blue) showed that XL-844 alone or in combination with radiation induced an uneven chromatid distribution along with atypical α-tubulin (which was co-stained with red) (white arrow-head, Fig. 5a). Quantification of the number of cells with α-tubulin, γ-H2AX, and co-staining of both showed that radiation treatment induced a small increase in the number of cells stained with α-tubulin with mostly normal bipolar anatomy (7% in controls and 10% with 6 Gy of radiation). Interestingly, combined treatment with XL-844 Gy and 6 Gy of radiation dramatically increased the number of α-tubulin-positive cells from 7% to 18% (Fig. 5b), and the localization of α-tubulin was not confined to microtubular spindles and polar bodies, but was also observed as irregular staining independent of polar bodies. When the images of samples stained with each of these two markers, γ-H2AX and α-tubulin, were merged, it was evident that most of the cells showing aberrant α-tubulin staining also stained positive for pan-nuclear γ-H2AX (Fig. 5b).

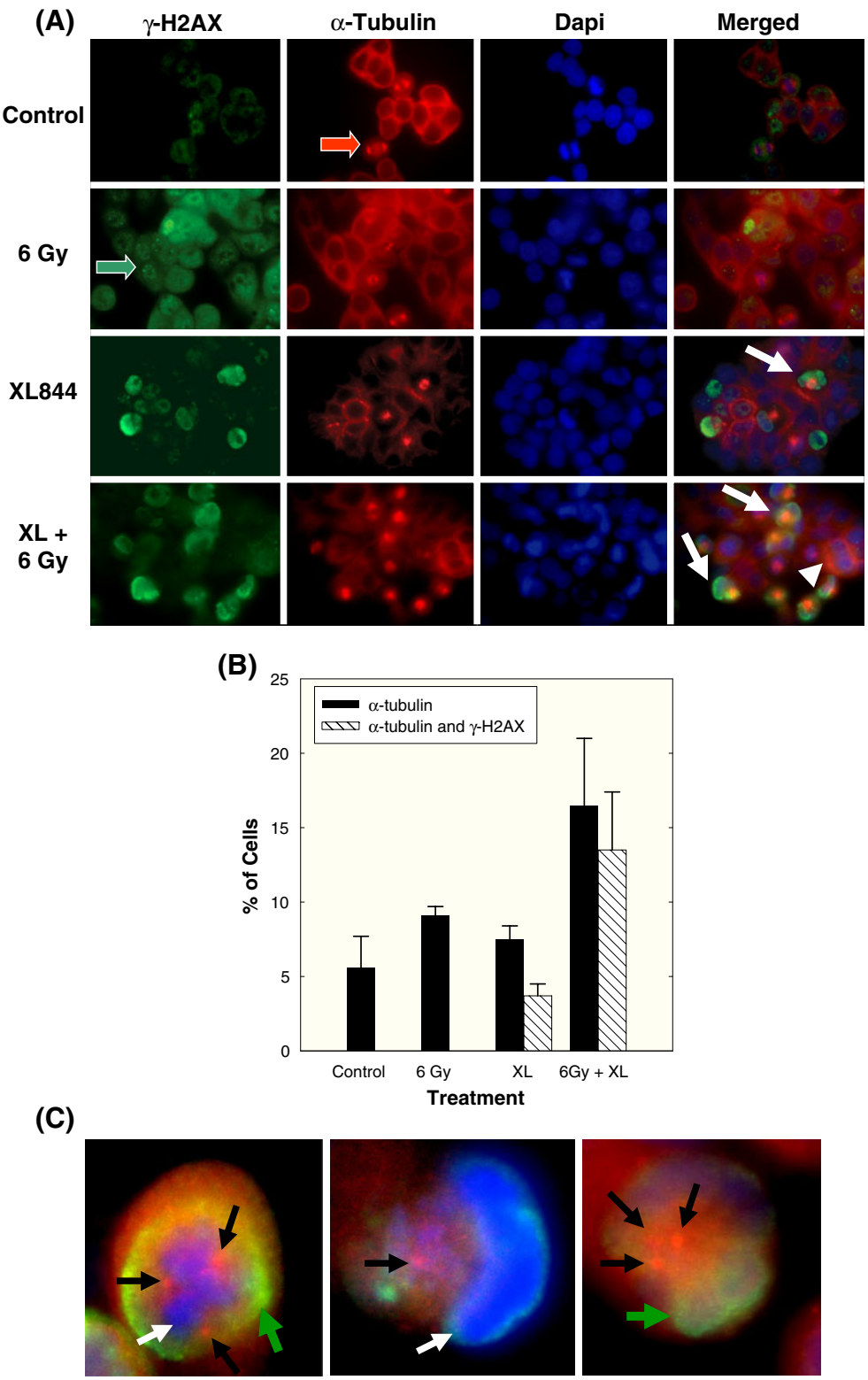

Fig. 5 Immuno-cytochemical analysis for γ-H2AX, α-tubulin and γ-tubulin staining. **a** Cells were co-stained for the expression of α-tubulin (*Texas-red*) and γ-H2AX (*FITC-green*) at 16 h after treatment with 6 Gy, XL-844, or both (irradiation followed by XL-844 treatment). DNA is stained using Dapi (*blue*). Red arrow: α-tubulin staining (*red*), normal interpolar spindles at anaphase; green arrow: γ-H2AX foci; white arrows: atypical α-tubulin staining (*red*) co-stained with pan-nuclear γ-H2AX (*green*). white thick arrow-head: co-staining of atypical α-tubulin (*red*) and DNA (*blue*), showing uneven chromatid distribution. **b** Quantification of nuclei positive for abnormal α-tubulin staining and co-staining of α-tubulin and pan-nuclear γ-H2AX. **c** Representative nuclei displaying signs of mitotic catastrophe with abnormal staining of γ-tubulin (*red stain*, *black arrows*), γ-H2AX (*green stain*, *green arrows*) and DNA (*blue stain*, *white arrows*) 16 h after the combined treatment with XL-844 and radiation

Treatment-induced mitotic catastrophe was further evident when the cells were co-stained with γ-tubulin and γ-H2AX. As shown in Fig. 5c, combination treatment evoked the appearance of other nuclear phenotypic changes representing mitotic catastrophe, such as formation of large cells, lobulated micronuclei, multi- or unipolar spindles (red stain, black arrows) and/or lagging chromosomal material (blue stain, white arrows). Interestingly, most of the nuclei

that exhibited, features of mitotic catastrophe also showed pan-nuclear γ-H2AX staining (green stain, green arrows).

Discussion

The present study explored the ability of XL-844, a novel Chk inhibitor, to enhance the sensitivity of HT-29 cells to irradiation and also investigated the underlying mechanisms therein. XL-844 improved cell radiosensitivity when administered immediately after irradiation. Neither pre-irradiation administration of XL-844 with discontinuation just before irradiation nor administration of XL-844 beginning 24 h after irradiation was effective in enhancing cell radiosensitivity, suggesting that XL-844 may enhance cell radiosensitivity by interfering with the cellular repair capacity immediately after irradiation. As an inhibitor of Chk kinases, XL-844 interferes with the interactions between Chk proteins and their downstream effector proteins, leading to premature cell progression through the cell-cycle to mitosis. In our model cell system, Chk2, rather than Chk1 appeared to be activated by irradiation that was suppressed by XL-844. This finding is consistent with reports of irradiation activating Chk2, and to a lesser extent, Chk1, in mammalian cells [8, 9, 18]. It is well established that Chk2 is phosphorylated and activated in response to DNA damage in an ATM-dependent manner [7, 19, 20]. Previous reports have shown that inhibition of Chk kinases successfully sensitizes human tumor cells to cytotoxic chemotherapy [21–23] and ionizing radiation [24–26]. Whereas, most chemical inhibitors are known to inhibit other kinases as well, siRNA and knockdown studies, targeting specifically Chk proteins, have established Chk kinases as promising therapeutic targets for improving tumor cell response to some cancer therapies.

Specific inhibitors of Chk kinases have been developed recently and found to exhibit synergistic effect when combined with some cytotoxic chemotherapeutic agents (see review, [16]). Mathews et al. [13] recently showed that XL-844 enhanced the cytotoxic effects of gemcitabine chemotherapy. Here we report for the first time that XL-844 enhanced human cancer cell radiosensitivity primarily via Chk2 inhibition. At the molecular level, XL-844 inhibited radiation-induced activation of Chk2, which led to activation of cdc2 further downstream and promoted the premature entry of cells into mitosis. The cdc2 kinase is inhibited downstream of Chk1/2 signaling, and activation of cdc2 requires a multistep process that includes phosphorylation of cdc2 at Thr-161 [27–30] after dephosphorylation at Tyr-15 [30, 31]. Our data showed that cdc2 was constitutively phosphorylated at Tyr-15 in HT-29 cells that was not affected by XL-844 and/or radiation. However, we found an increase in the level of cdc2 phosphorylated at Thr161 which is considered a step in the activation of cdc2. Thus, the underlying mechanisms of phosphorylation of cdc2 at dual sites (Tyr-15 as well as Thr-161) need further investigation.

Interestingly, XL-844, despite being a Chk inhibitor, did not inhibit phosphorylation of Chk1 (Ser-345), but rather promoted it when tested 30 min after drug exposure. This finding is consistent with that from a previous study demonstrating XL-844-induced phosphorylation of Chk1 (Ser-317) in PANC-1 cells [13]. However, it is not clear whether the phosphorylation of Chk1 at these sites indicates the activation of Chk1.

The mechanisms of XL-844-induced radiosensitization in our model system appeared to involve inhibition of DNA repair and induction of mitotic catastrophe. Inhibition of DNA repair is one of the major mechanisms by which radiosensitizing agents increase the radiation-induced cell killing in many tumor cell types [32, 33]. Early responses of cells to radiation-induced DNA damage include phosphorylation of H2AX at Ser-345 (γ-H2AX) and accumulation of γ-H2AX at the site of DSBs forming γ-H2AX foci. Upon initiation of repair of these DSBs, γ-H2AX undergoes dephosphorylation. Prolongation of the presence of γ-H2AX in the nucleus indicates the presence of unrepaired DSBs that may lead to cell death. Treatment with XL-844 either alone or in combination with 6 Gy of radiation induced appearance of massive levels of pan-nuclear γ-H2AX and irradiation-induced γ-H2AX foci. Similar pan-nuclear γ-H2AX expression was reported previously after treatment with another Chk inhibitor, CEP-3891, in human U2-OS osteosarcoma cells [34].

Staining of tubulin subunits showed that XL-844 and radiation treatments resulted in an increase in the number of cells expressing atypically distributed tubulin subunits. Such expression of tubulin subunits has been described in cells undergoing mitotic catastrophe [35–38]. The induction of mitotic catastrophe with abnormal mitotic cells [39–41] may result in apoptosis or other forms of cell death [34, 42, 43]. Mitotic spindle apparatus constitutes microtubules with α- and β-tubulin subunits and centrosomes with γ-tubulin [44, 45]. Our data on co-staining of tubulin subunits (α or γ) and γ-H2AX after treatments with XL-844 and radiation showed the presence of damaged DNA in mitotic cells (co-stained with α-tubulin) undergoing mitotic catastrophe as shown by an abnormal number of centrosomes (co-stained with γ-tubulin). Consistent with our results, Castedo et al [39] showed that Chk2 was a negative regulator of mitotic catastrophe in Hela and HT116 cells. Chemical inhibition of Chk2 or transfection with a dominant-negative Chk2 mutant induced mitotic catastrophe and sensitized proliferating cells to doxorubicin [39].

In conclusion, this is the first study demonstrating in vitro sensitization to the effects of radiation by XL-844.

The mechanism of radiosensitization included inhibition of DNA repair and induction of premature mitotic entry, leading to mitotic catastrophe. These data suggest that XL-844 has the potential to increase tumor response to radiotherapy and warrants further investigation using in vivo tumor models.

Acknowledgements This work was supported partly by Exelixis Inc., CA, USA (PI: U. Raju) and partly by a UICC ACSBI fellowship supplemented by the University of Zurich (O. Riesterer). We thank Peter Lamb Ph.D. (Exelixis Incorporation, San Francisco, CA) for his involvement in designing the experiments.

References

1. Zhou BB, Elledge SJ (2000) The DNA damage response: putting checkpoints in perspective. Nature 408(6811):433–439
2. Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y, Ziv Y (1998) Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science 281(5383):1674–1677
3. Canman CE, Lim DS, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, Appella E, Kastan MB, Siliciano JD (1998) Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science 281(5383):1677–1679
4. Elledge SJ (1996) Cell cycle checkpoints: preventing an identity crisis. Science 274(5293):1664–1672
5. Painter RB, Young BR (1980) Radiosensitivity in ataxia-telangiectasia: a new explanation. Proc Natl Acad Sci USA 77 (12):7315–7317
6. Samuel T, Weber HO, Funk JO (2002) Linking DNA damage to cell cycle checkpoints. Cell Cycle 1(3):162–168
7. Chaturvedi P, Eng WK, Zhu Y, Mattern MR, Mishra R, Hurle MR, Zhang X, Annan RS, Lu Q, Faucette LF, Scott GF, Li X, Carr SA, Johnson RK, Winkler JD, Zhou BB (1999) Mammalian Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway. Oncogene 18(28):4047–4054
8. Pommier Y, Sordet O, Rao VA, Zhang H, Kohn KW (2005) Targeting chk2 kinase: molecular interaction maps and therapeutic rationale. Curr Pharm Des 11(22):2855–2872
9. Bartek J, Falck J, Lukas J (2001) CHK2 kinase—a busy messenger. Nat Rev Mol Cell Biol 2(12):877–886
10. Taylor WR, Stark GR (2001) Regulation of the G2/M transition by p53. Oncogene 20(15):1803–1815
11. Choudhury A, Cuddihy A, Bristow RG (2006) Radiation and new molecular agents part I: targeting ATM-ATR checkpoints, DNA repair, and the proteasome. Semin Radiat Oncol 16(1):51–58
12. Xu B, Kim ST, Lim DS, Kastan MB (2002) Two molecularly distinct G(2)/M checkpoints are induced by ionizing irradiation. Mol Cell Biol 22(4):1049–1059
13. Matthews DJ, Yakes FM, Chen J, Tadano M, Bornheim L, Clary DO, Tai A, Wagner JM, Miller N, Kim YD, Robertson S, Murray L, Karnitz LM (2007) Pharmacological abrogation of S-phase checkpoint enhances the anti-tumor activity of gemcitabine in vivo. Cell Cycle 6(1):104–110
14. Busby EC, Leistritz DF, Abraham RT, Karnitz LM, Sarkaria JN (2000) The radiosensitizing agent 7-hydroxystaurosporine (UCN-01) inhibits the DNA damage checkpoint kinase hChk1. Cancer Res 60 (8):2108–2112
15. Jobson AG, Lountos GT, Lorenzi PL, Llamas J, Connelly J, Cerna D, Tropea JE, Onda A, Zoppoli G, Kondapaka S, Zhang G, Caplen NJ, Cardellina JH, Yoo SS, Monks A, Self C, Waugh DS, Shoemaker RH, Pommier Y (2009) Cellular inhibition of Chk2 kinase and potentiation of camptothecins and radiation by the novel Chk2 inhibitor PV1019. J Pharmacol Exp Ther Epub ahead of print
16. Ashwell S, Janetka JW, Zabludoff S (2008) Keeping checkpoint kinases in line: new selective inhibitors in clinical trials. Expert Opin Investig Drugs 17(9):1331–1340
17. Fertil B, Malaise EP (1981) Inherent cellular radiosensitivity as a basic concept for human tumor radiotherapy. Int J Radiat Oncol Biol Phys 7(5):621–629
18. Antoni L, Sodha N, Collins I, Garrett MD (2007) CHK2 kinase: cancer susceptibility and cancer therapy—two sides of the same coin? Nat Rev Cancer 7(12):925–936
19. Matsuoka S, Huang M, Elledge SJ (1998) Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science 282 (5395):1893–1897
20. Ahn JY, Schwarz JK, Piwnica-Worms H, Canman CE (2000) Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. Cancer Res 60(21):5934–5936
21. Tse AN, Rendahl KG, Sheikh T, Cheema H, Aardalen K, Embry M, Ma S, Moler EJ, Ni ZJ, Lopes de Menezes DE, Hibner B, Gesner TG, Schwartz GK (2007) CHIR-124, a novel potent inhibitor of Chk1, potentiates the cytotoxicity of topoisomerase I poisons in vitro and in vivo. Clin Cancer Res 13(2 Pt 1):591–602
22. Xiao Z, Xue J, Sowin TJ, Rosenberg SH, Zhang H (2005) A novel mechanism of checkpoint abrogation conferred by Chk1 down-regulation. Oncogene 24(8):1403–1411
23. Ganzinelli M, Carrassa L, Crippa F, Tavecchio M, Broggini M, Damia G (2008) Checkpoint Kinase 1 down-regulation by an inducible small interfering RNA expression system sensitized in vivo tumors to treatment with 5-fluorouracil. Clin Cancer Res 14 (16):5131–5141
24. Koniaras K, Cuddihy AR, Christopoulos H, Hogg A, O'Connell MJ (2001) Inhibition of Chk1-dependent G2 DNA damage checkpoint radiosensitizes p53 mutant human cells. Oncogene 20(51):7453–7463
25. Zhao H, Watkins JL, Piwnica-Worms H (2002) Disruption of the checkpoint kinase 1/cell division cycle 25A pathway abrogates ionizing radiation-induced S and G2 checkpoints. Proc Natl Acad Sci USA 99(23):14795–14800
26. Yu Q, La Rose J, Zhang H, Takemura H, Kohn KW, Pommier Y (2002) UCN-01 inhibits p53 up-regulation and abrogates gamma-radiation-induced G(2)-M checkpoint independently of p53 by targeting both of the checkpoint kinases, Chk2 and Chk1. Cancer Res 62(20):5743–5748
27. Atherton-Fessler S, Parker LL, Geahlen RL, Piwnica-Worms H (1993) Mechanisms of p34cdc2 regulation. Mol Cell Biol 13 (3):1675–1685
28. Galaktionov K, Lee AK, Eckstein J, Draetta G, Meckler J, Loda M, Beach D (1995) CDC25 phosphatases as potential human oncogenes. Science 269(5230):1575–1577
29. Norbury C, Nurse P (1992) Animal cell cycles and their control. Annu Rev Biochem 61:441–470
30. Watanabe N, Broome M, Hunter T (1995) Regulation of the human WEE1Hu CDK tyrosine 15-kinase during the cell cycle. Embo J 14(9):1878–1891
31. Hunter T (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell 80(2):225–236
32. Kastan MB, Lim DS, Kim ST, Yang D (2001) ATM—a key determinant of multiple cellular responses to irradiation. Acta Oncol 40(6):686–688
33. Raju U, Ariga H, Dittmann K, Nakata E, Ang KK, Milas L (2005) Inhibition of DNA repair as a mechanism of enhanced radio-response of head and neck carcinoma cells by a selective

cyclooxygenase-2 inhibitor, celecoxib. Int J Radiat Oncol Biol Phys 63(2):520–528
34. Syljuasen RG, Sorensen CS, Hansen LT, Fugger K, Lundin C, Johansson F, Helleday T, Sehested M, Lukas J, Bartek J (2005) Inhibition of human Chk1 causes increased initiation of DNA replication, phosphorylation of ATR targets, and DNA breakage. Mol Cell Biol 25(9):3553–3562
35. Belmont LD, Hyman AA, Sawin KE, Mitchison TJ (1990) Real-time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts. Cell 62(3):579–589
36. Eriksson D, Lofroth PO, Johansson L, Riklund KA, Stigbrand T (2007) Cell cycle disturbances and mitotic catastrophes in HeLa Hep2 cells following 2.5 to 10 Gy of ionizing radiation. Clin Cancer Res 13(18 Pt 2):5501s–5508s
37. McIntosh JR (1984) Cell biology.Microtubule catastrophe. Nature 312(5991):196–197
38. Robinson HM, Black EJ, Brown R, Gillespie DA (2007) DNA mismatch repair and Chk1-dependent centrosome amplification in response to DNA alkylation damage. Cell Cycle 6(8):982–992
39. Castedo M, Perfettini JL, Roumier T, Yakushijin K, Horne D, Medema R, Kroemer G (2004) The cell cycle checkpoint kinase Chk2 is a negative regulator of mitotic catastrophe. Oncogene 23 (25):4353–4361
40. Roninson IB, Broude EV, Chang BD (2001) If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. Drug Resist Updat 4(5):303–313
41. Cenciarelli C, Tanzarella C, Vitale I, Pisano C, Crateri P, Meschini S, Arancia G, Antoccia A (2008) The tubulin-depolymerising agent combretastatin-4 induces ectopic aster assembly and mitotic catastrophe in lung cancer cells H460. Apoptosis 13(5):659–669
42. Brown JM, Attardi LD (2005) The role of apoptosis in cancer development and treatment response. Nat Rev Cancer 5(3):231–237
43. Niida H, Tsuge S, Katsuno Y, Konishi A, Takeda N, Nakanishi M (2005) Depletion of Chk1 leads to premature activation of Cdc2-cyclin B and mitotic catastrophe. J Biol Chem 280(47):39246–39252
44. Mitchison T, Kirschner M (1984) Dynamic instability of microtubule growth. Nature 312(5991):237–242
45. Oakley CE, Oakley BR (1989) Identification of gamma-tubulin, a new member of the tubulin superfamily encoded by mipA gene of Aspergillus nidulans. Nature 338(6217):662–664