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Aurora kinase inhibition downregulates NF-κB and sensitises tumour cells to chemotherapeutic agents

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Abstract

We have identified that Aurora-A activates NF-κB via IκBα phosphorylation. Here, we analysed different human tumour cell types for their NF-κB activity. We found that there is an association between cell resistance to chemotherapeutic agents and NF-κB activation. A549 human lung adenocarcinoma cells and SKOV3 human ovarian cancer cells have high levels of NF-κB and are resistant to cytotoxic agents such as adriamycin and VP-16 (etoposide). We also found that in A549 and SKOV3 cells treated with a small molecule inhibitor towards Aurora kinases, the NF-κB activity was downregulated and the efficacy of cytotoxic drugs was enhanced. In addition, the transcriptional targets Bcl-X_L and Bcl-2 were downregulated. This study provides evidence for a potential mechanism of chemoresistance and may be useful for the enhancement of certain chemotherapeutics regimens.

Keywords: Aurora; NF-κB; Chemotherapeutic agents

The *Aurora-A* gene encodes a centrosome-associated, cell cycle regulated serine/threonine kinase [1,2]. Aurora-A is the human homolog of the Aurora protein kinase from *Drosophila* and Ipl1 kinase from *Saccharomyces cerevisiae* [3,4], and is a member of a family of Aurora kinases that includes Aurora-B and Aurora-C [2,5]. The *Aurora-A* gene encodes a 403 aa protein, and is located on human chromosome 20*q*13—a region that is amplified in a variety of human tumours [6], including 50% of primary colorectal cancers, in 6–18% of primary breast cancers, as well as in breast, ovarian, colon, and prostate tumour cell lines [2,7]. Ectopic expression of *Aurora-A* in murine fibroblasts results in abnormal centrosome amplification and cellular transformation [2,7]. *Aurora-A* is a low-penetrance tumour-susceptibility gene and the F31I polymorphism was reported to be associated with aneuploidy in colon cancer [8]. The F31I variant is also preferentially amplified in a variety of human cancers such as esophageal, breast, ovarian, lung, and prostate [9]. The overexpression of Aurora-A kinase and the association with genetic instability in tumours have made it the focus of drug discovery efforts aimed at the identification of small molecule inhibitors.

Recently, work from our laboratory has identified that Aurora-A regulates NF-κB via IκBα phosphorylation (submitted data). NF-κB is activated in certain cancers as well as in response to chemotherapy and radiation [10]. NF-κB activation implicates the IκB kinase complex (IKK-α, IKK-β, and NEMO) which phosphorylates IκB proteins that sequester NF-κB in the cytoplasm. Phosphorylated IκB is targeted for proteolysis and results in nuclear translocation and activation of NF-κB complexes [11–13]. A vast majority of studies have shown that NF-κB plays a key role in regulation of cell proliferation, inflammation, angiogenesis, and suppression of apoptosis through its signalling [11–15]. While activation of NF-κB may induce apoptosis in certain situations, most data suggest that NF-κB mediates survival signals that counteract apoptosis

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[16,17]. It has been reported that constitutively activated NF-κB may be critical in the development of drug resistance in tumour cells [18]. Therefore, agents that are able to downregulate NF-κB activity might be considered as an auxiliary approach in combination with chemotherapy for a variety of cancers. These considerations led us to examine whether regulation of NF-κB signalling by Aurora-A inhibition could sensitise cancer cells to chemotherapeutic drugs and also to study potential mechanisms by which chemoresistance can be overcome.

Materials and methods

Reagents. VP-16 (etoposide) was purchased from Calbiochem (Nottingham, UK). Adriamycin and cisplatin were purchased from Sigma (Dorset, UK). VX-680 was kindly provided by Chroma, Oxford, UK. Anti-Bcl-X_L and anti-PARP antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-Bcl-2 antibody was obtained from Calbiochem (Nottingahm, UK). Transfection reagent Lipofectamine 2000 was purchased from Invitrogen (Paisley, UK). The kits of Duel-Luciferase® Reporter Assay System were purchased from Promega (Madison, WI).

Cell cultures. Ovarian (OVCAR8, SKOV3), lung (A549), colon (SW620, HT29, SW480, HCT116, HCT116p53−/−, Colo205), breast (MDA-MB-231, T47D, MCF7), osteosarcoma (Saos2), and cervical (HeLa) tumour cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and maintained in a humidified atmosphere at 37 °C with 5% CO_2.

NF-κB luciferase reporter assays. For NF-κB luciferase assays, cells were seeded at 70% confluency in 6-well plates and transfected with Lipofectamine 2000 reagent according to manufacturer's instructions (Invitrogen). Cells were transiently transfected with 0.25 μg/well of NF-κB-dependent luciferase reporter plasmid 3×κBL. For the luciferase assay, cells were lysed in Reporter lysis buffer (Promega) and activity was measured with luciferase assay reagent (Promega) according to manufacturer's instructions. Normalisation for transfection efficiency was done by co-transfecting 500 ng of a β-galactosidase expression plasmid (pGK-β-gal) and measuring β-galactosidase activity. Relative luciferase activities are expressed as fold of activation over the activity of NF-κB-dependent luciferase reporter alone and were calculated by dividing the values of luciferase activity with the values for β-galactosidase activity.

Cytotoxicity assay. The effects of chemotherapeutic agents on cellular proliferation were determined using the MTT assay according to manufacturer's instructions (Sigma). Briefly, the cells were seeded in triplicate into 96-well plates at 2500 cells/well 24 h before treatment with a range of concentrations of chemotherapeutic agents for the indicated time periods. At each time point, 15 μl/well of 0.5% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the cells. After 4 h incubation at 37 °C, the dye-stained viable cells were extracted by adding 200 μl/well of dimethyl sulphoxide (DMSO) into each well. The optical density was measured at 570 nm using the Wallac VICTOR$^2_{TM}$ 1420 Multilabel Counter (Perkin-Elmer).

Western blot analyses. Western blot analysis was performed according to standard procedures. At the indicated time points of treatments, the cells were lysed in lysis buffer (25 mM Hepes, 135 mM NaCl, 1% NP40, 5 mM EDTA, 1 mM EGTA, 1 mM $ZnCl_2$, 50 mM sodium fluoride (NaF), 10% glycerol, 2 mM PMSF, and 1 mM sodium orthovanadate supplemented with protease inhibitor cocktail set III (Calbiochem)). Lysates were then extracted by centrifuging at 13,200 rpm for 20 min on a desktop centrifuge. Thirty micrograms of cell extract protein was loaded and separated via sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 7.5–12% gel, transferred to PVDF membrane (Millipore), and probed with primary antibodies against Bcl-2, Bcl-X_L, and PARP. Following incubation at 4 °C overnight, the blots were probed with peroxidase-labeled goat anti-rabbit secondary antibody (Dako Cytomation). The membranes were then developed with ECL Western blotting detection reagents (Amersham International) followed by autoradiography.

Results

We have recently identified that Aurora-A regulates NF-κB activity via IκBα phosphorylation (submitted data). Inhibition of Aurora-A by siRNA or small molecule inhibitors led to NF-κB downregulation. We first screened breast tumour cell lines for active NF-κB and found that in MCF7 cells, the basal endogenous NF-κB activity was increased compared to MDA-MB-231 (Fig. 1A and B). Inhibition of Aurora-A into MCF7 and MDA-MB-231 cells treated with the Aurora kinase inhibitor VX-680

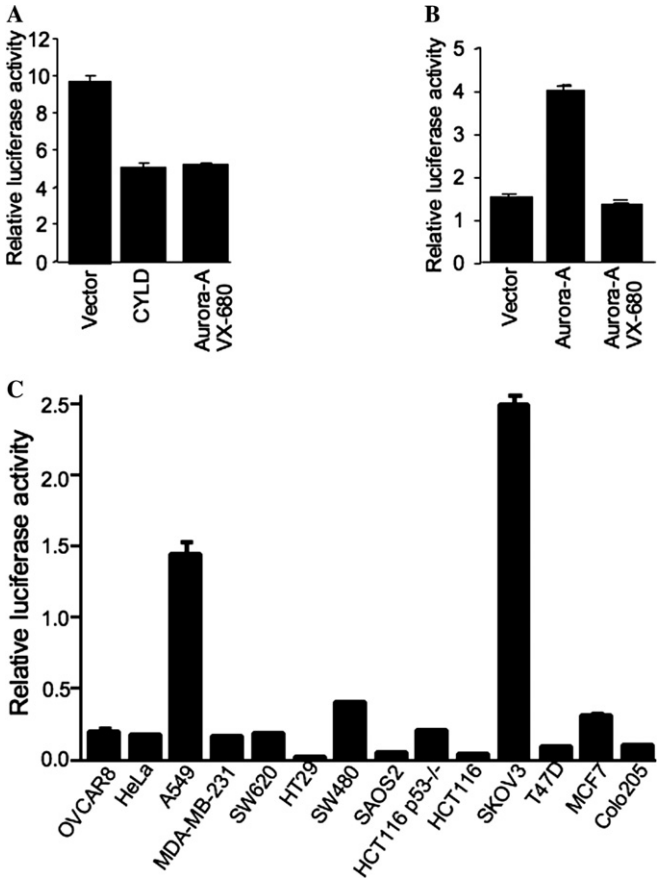

Fig. 1. NF-κB activity in human tumour cell lines. (A) MCF7 cells were transfected with the 3×κBL and the pGK-β-galactosidase reporter plasmids together with plasmid expressing the NF-κB repressor CYLD or treated with the Aurora kinase inhibitor VX-680. Luciferase activity of endogenous NF-κB was assayed. (B) MBA-MD-231 cells were transfected with vector DNA, plasmid expressing Aurora-A or treated with the Aurora kinase inhibitor VX-680 together with NF-κB luciferase reporter plasmid. Luciferase activity was estimated as in (A). (C) Reporter gene analysis of OVCAR8, SKOV3, A549, SW620, HT29, SW480, HCT116, HCT116p53−/−, Colo205, MDA-MB-231, T47D, MCF7, Saos2, and HeLa cells, transiently cotransfected with the 3×κBL and the pGK-β-galactosidase reporter plasmids and assayed for NF-κB-dependent luciferase and β-galactosidase activation. Data are mean values of three independent experiments.

[19], suppressed both the constitutively activated (MCF7; Fig. 1A) or the Aurora-A-mediated (MDA-MB-231; Fig. 1B) NF-κB gene transactivation as did as a positive control, expression of the cylindromatosis (CYLD) [20] deubiquitinating enzyme (Fig. 1A). To determine the NF-κB activity in a panel of human tumour cell lines we measured the levels of activated NF-κB in extracts from ovarian (OVCAR8, SKOV3), lung (A549), colon (SW620, HT29, SW480, HCT116, HCT116p53−/−, Colo205), breast (MDA-MB-231, T47D, MCF7), osteosarcoma (Saos2), and cervical (HeLa) tumour cell lines by luciferase assay. Tumour cells were transiently transfected with the NF-κB-dependent luciferase reporter plasmid 3×κBL. Normalization for transfection efficiency was done by cotransfecting the β-galactosidase expression plasmid (pGK-β-gal) and measuring β-galactosidase activity. The results showed that the luciferase activity, which represents the transcriptional activation of NF-κB, was elevated in SKOV3 ovarian cancer and A549 lung cancer cell lines indicating constitutively activated NF-κB in these two cell lines (Fig. 1C). Several studies have been demonstrated that A549 and SKOV3 cell lines are resistant to certain chemotherapeutic drugs such as cisplatin, adriamycin and VP-16 [21,22].

Inhibitory effects of VX-680 [19] on NF-κB activity was examined by a reporter assay using A549 and SKOV3 cells transfected with the NF-κB-dependent luciferase reporter plasmid 3×κBL. Luciferase activity was reduced by treatment with VX-680 for 24 h. A549 cells showed a dose-dependent downregulation of NF-κB activity (Fig. 2A) whereas, SKOV3 cells showed downregulation of NF-κB at a concentration of 600 nM of VX-680 when treated for 24 h (Fig. 2B). In a parallel experiment A549 and SKOV3 cells were treated with the same concentrations of VX-680 for 24 h as above and allowed to grow for an additional 24 h. This experiment was carried out to estimate the concentration of VX-680 that shows the maximal inhibition of NF-κB activity with minimal cell death (⩽5%). The corresponding concentrations of VX-680 were found to be 10 μM for A549 and 800 nM for SKOV3 (data not shown).

It has been shown that A549 cells are resistant to cytotoxic effects of agents such as adriamycin, VP-16, cisplatin, and TNF-α [21]. This resistance was due to upregulated expression of Bcl-X_L and/or Bfl-1/A1 through an NF-κB-dependent pathway. While parental A549 cells were resistant to the cytotoxic effects of both TNF-α and chemotherapy agents, NF-κB-blocked A549 cells were sensitized to both. Moreover, expression of Bcl-X_L in the NF-κB deficient cells provided protection against chemotherapeutic treatment.

To test for the ability of these cells to resist the effect of adriamycin, VP-16, and cisplatin treatment, A549 cells were treated with the above agents and cell viability and cell death were assessed using the MTT assay. A549 cells remained viable (roughly 90% of the cell population) when treated with adriamycin, VP-16 and cisplatin (Fig. 3A and B and data not shown). To explore the possibility that downregulation of NF-κB signaling via inhibition of Aurora kinases would sensitize A549 cells to chemotherapy-mediated cell death, we compared the ability of the three commonly used chemotherapy agents to differentially affect the cell viability of A549 cells with and without treatment with the Aurora kinase inhibitor VX-680. In all three treatments, parental A549 cells were resistant to the pro-death effects of chemotherapeutic agents, as assessed by MTT assay. In contrast, the NF-κB downregulated A549 cells treated with VX-680 showed increased sensitivity to adriamycin (Fig. 3A) and VP-16 (Fig. 3B) but not to cisplatin (data not shown). One of the causes of drug resistance is known to be upregulation of antiapoptotic markers such as Bcl-X_L. This effect requires signaling through NF-κB. To determine whether the sensitization of A549 to chemotherapeutic agents was due to the downregulation of NF-κB and therefore, of Bcl-X_L, we examined the levels of

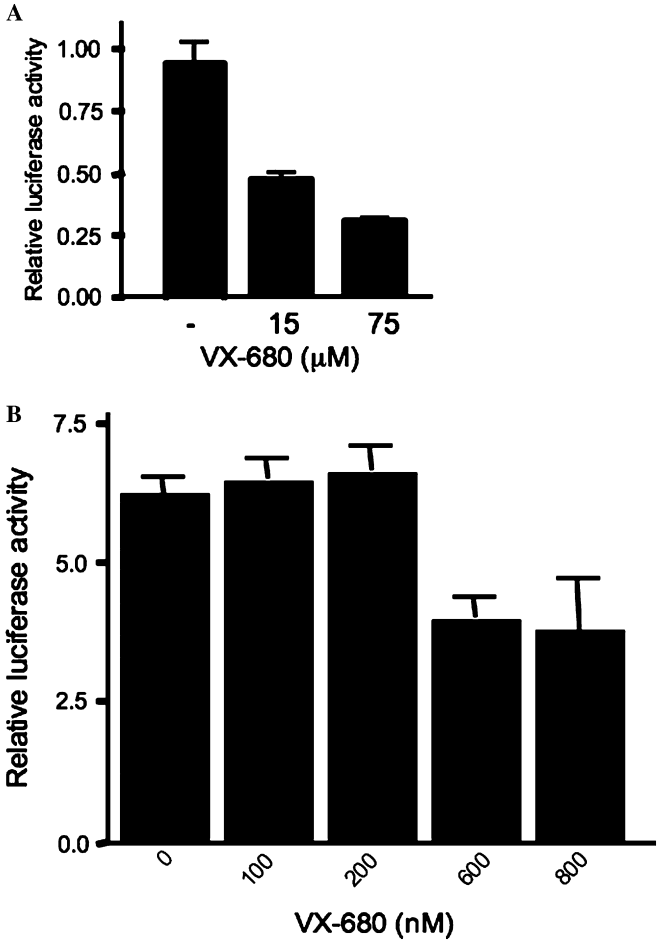

Fig. 2. NF-κB activity in A549 and SKOV3 cell lines treated with VX-680. (A) A549 cells were seeded in triplicate and different concentrations of VX-680 ($1 \times IC_{50} = 15$ μM and $5 \times IC_{50} = 75$ μM) were added for 24 h. The cells were transiently cotransfected with the 3×κBL and the pGK-β-galactosidase reporter plasmids and 24 h later were assayed for NF-κB-dependent luciferase activation. (B) SKOV3 cells were seeded in triplicate and different concentrations of VX-680 were added for 24 h. The cells were transiently cotransfected with the 3×κBL and the pGK-β-galactosidase reporter plasmids and 24 h later were assayed for NF-κB-dependent luciferase activation. Data are mean values of three independent experiments.

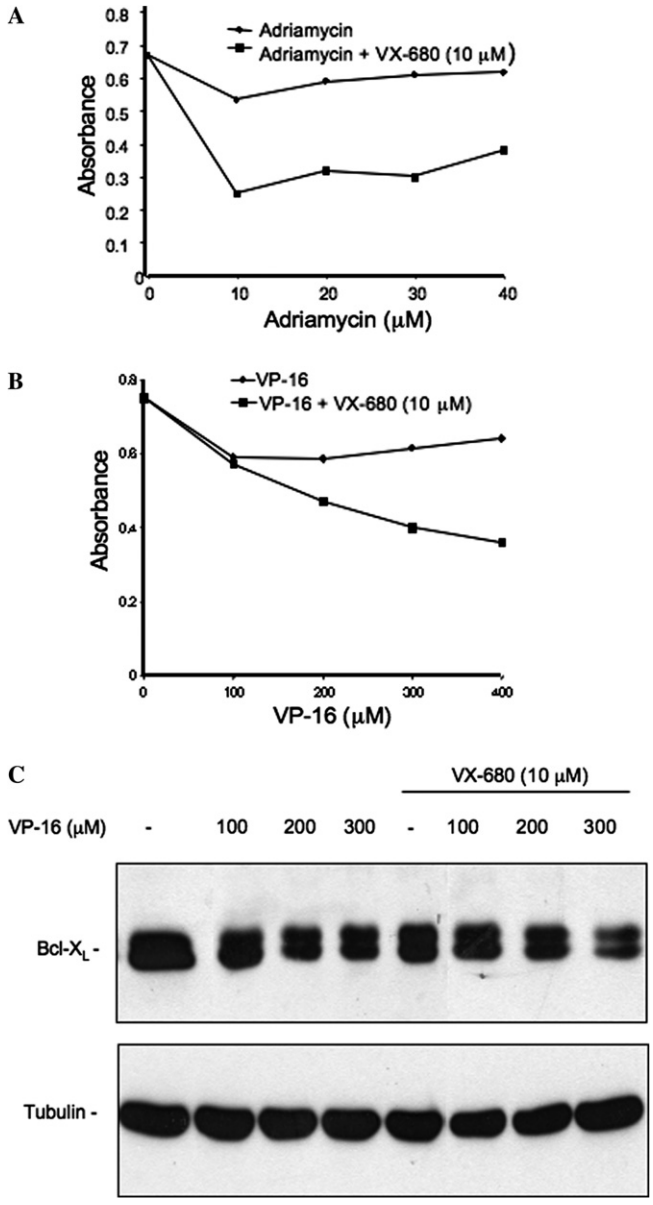

Fig. 3. Downregulation of NF-κB activity by VX-680 sensitizes A549 cells to adriamycin and VP-16-induced cell death. (A) Treated and untreated A549 cells with VX-680 were exposed to the indicated concentrations of adriamycin for 24 h and cell viability was then assessed by MTT assay. (B) In treated and untreated A549 cells with VX-680 the indicated concentrations of VP-16 were added for 24 h and the cell viability was assessed by MTT assay. (C) Treated and untreated A549 cells with VX-680, followed by addition of the indicated concentrations of VP-16, were assayed by Western blot using anti-Bcl-X_L specific antibody. Sample loading was normalized by detection of the levels of tubulin protein.

Bcl-X_L in A549 cells treated and untreated with VX-680. As Fig. 3C shows, only in A549 cells treated with VX-680 followed by exposure to different concentrations of VP-16, the levels of Bcl-X_L were reduced in a dose-dependent manner. Thus, indicating that the mechanism of chemo-resistance of A549 is due at least in part from the antiapoptotic effect of NF-κB-induced Bcl-X_L.

We next examined whether inhibition of constitutively activated NF-κB in SKOV3 cells would increase the efficacy of VP-16 induced apoptosis. We treated SKOV3 cells with VX-680 for 24 h, followed by addition of increasing concentrations of VP-16. We assessed the cell death using a MTT assay as described above. VP-16 and VX-680-treated SKOV3 cells showed up to five times lower IC_{50} value to VP-16 alone, suggesting that treatment with the Aurora kinase inhibitor and therefore, downregulation of NF-κB activity, increases the efficacy of cell death by VP-16 (Fig. 4A). Moreover, the downregulation of NF-κB led to the acceleration of the apoptotic program, as PARP cleavage was markedly increased in VX-680-treated cells (Fig. 4B). To confirm that increased VP-16 sensitivity in Aurora-A-inhibited cells was due to NF-κB downregulation we examined the levels of Bcl-2 in treated and untreated cells. As Fig. 4C shows, Bcl-2 is downregulated in a dose-dependent manner in SKOV3 cells treated with VX-680.

Discussion

NF-κB is a key transcription factor involved in the expression of genes that play key roles in growth, oncogen-

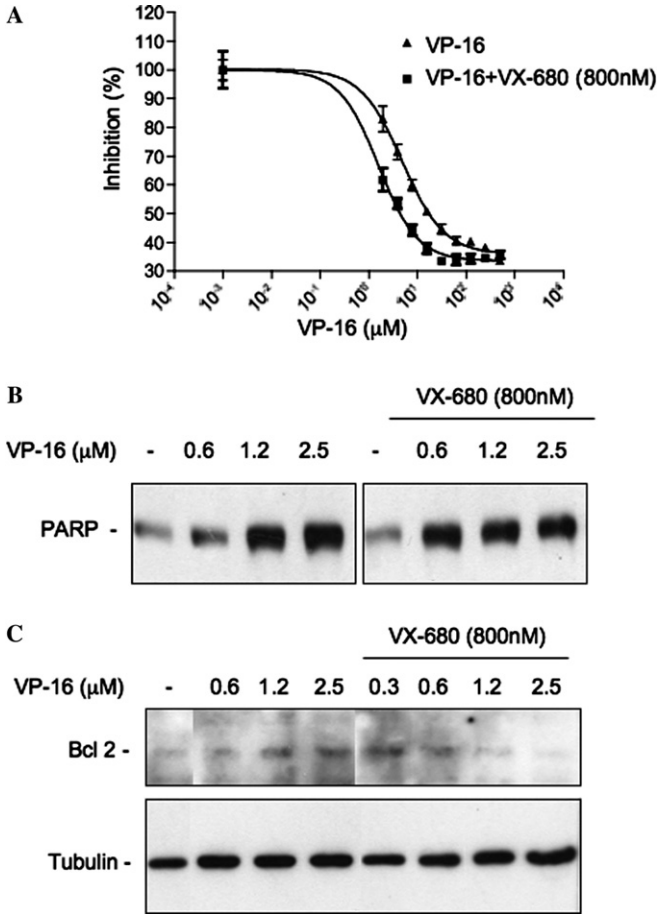

Fig. 4. Downregulation of NF-κB activity by VX-680 sensitizes SKOV3 cells to VP-16. (A) Treated and untreated SKOV3 cells with VX-680 were exposed to the indicated concentrations of VP-16 for 24 h and cell viability was then assessed by MTT assay. In SKOV3 cells and SKOV3 cells treated with VX-680 the indicated concentrations of VP-16 were added for 24 h. Cleaved PARP (B) and Bcl-2 levels (C) were assessed via Western blot analysis. Tubulin provides a loading control.

esis, differentiation, apoptosis, tumourigenesis and immune and inflammatory responses [11,12]. NF-κB activity is stimulated in response to a variety of stimuli such as DNA damage, cytokines and chemotherapeutic reagents [10]. The role of NF-κB in chemotherapeutic drug resistance has been associated with the induction of survival signals through the upregulation of anti-apoptotic genes such as Bcl-X_L [23]. Therefore, its constitutive activity has been suggested to promote drug resistance in cancer cells [24–26]. Inhibition of its survival signaling would therefore, sensitize cells to chemotherapy-dependent apoptosis facilitating the efficacy of the drug.

We have recently identified that Aurora-A is regulating NF-κB activity via IκBα phosphorylation (submitted data). Inhibition of endogenous Aurora-A reduces TNFα-induced IκBα degradation. Samples (13.6%) of primary human breast cancers showed *Aurora-A* gene amplification, all of which exhibited nuclear localisation of NF-κB. We also showed that Aurora-A-depleted HeLa cells treated with cisplatin severely suppressed NF-κB activation. Moreover, the downregulation of NF-κB led to the acceleration of the apoptotic program, as PARP cleavage was markedly increased. Constitutive activation of NF-κB has been described in a great number of solid tumours, and this activation appears to support cancer cell survival and to reduce the sensitivity to chemotherapeutic drugs. In order to examine whether Aurora-A-mediated regulation of NF-κB could affect the efficacy of certain chemotherapeutic agents we initially examined the levels of NF-κB activity in a panel of human tumour cell lines. The results showed that A549 lung cancer and SKOV3 ovarian cancer cell lines exhibit high levels of constitutively activated NF-κB. In a series of studies, it has been shown that A549 cells are resistant to certain chemotherapeutic agents such as cisplatin, adriamycin, TNF-α and VP-16 (etoposide) [21]. In order to investigate whether this Aurora-A-mediated NF-κB inhibition could sensitize A549 cells to chemotherapeutic agents, we first treated the cells with the Aurora kinase inhibitor VX-680 followed by addition of the particular agent. We showed that whereas A549 cells are resistant to cisplatin, adriamycin and VP-16, VX-680-treated A549 cells had increased sensitivity to both adriamycin and VP-16. Thus, it appears that constitutive activation of NF-κB mediates resistance to these chemotherapeutic drugs in A549 cells and inhibition of NF-κB activation sensitizes these cells to adriamycin and VP-16. Interestingly, A549 cells retained the resistance to cisplatin showing that this effect is potentially due to a pathway distinct to NF-κB. In the SKOV3 ovarian tumour cell line pre-treatment with the Aurora kinase inhibitor reduced the IC_{50} concentration of VP-16 up to five times. Moreover, PARP cleavage, a cellular marker for the induction of the apoptotic program, was markedly increased in Aurora kinase inhibited cells.

How does Aurora kinase-dependent downregulation of NF-κB cause the sensitization of A549 and SKOV3 cells to chemotherapeutic agents? It was reported that NF-κB inhibition diminished the expression of pro-survival genes regulated by NF-κB, such as Bcl-X_L and Bcl-2. We also showed that NF-κB downregulation by Aurora kinase inhibition caused reduction of the expression of these survival genes in A549 and SKOV3 cells. Collectively, our findings may have important contributions for cancer chemotherapy. Aurora-A inhibition enhances the efficacy of chemotherapeutic agents and reverses acquired resistance resulting from the activation of NF-κB. Consequently, preventing NF-κB activation, by inhibition of Aurora-A, may provide a valuable enhancement to specific chemotherapeutic regimens.

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