Oncogene (2001) 20, 6048–6056

www.nature.com/onc

Increased and correlated nuclear factor-kappa B and Ku autoantigen activities are associated with development of multidrug resistance

Jee Hyun Um[1,2,4], Chi Dug Kang[1,2,4], Bok Gun Lee[1], Dong Wan Kim[3], Byung Seon Chung[1] and Sun Hee Kim*[,1]

[1]*Department of Biochemistry, College of Medicine, Pusan National University, Pusan 602-739, South Korea;* [2]*Research Center for Molecular Medicine, College of Medicine, Pusan National University, Pusan 602-739, South Korea;* [3]*Department of Microbiology, College of Natural Sciences, Chang Won National University, Chang Won 641-773, South Korea*

In this study, we investigated possible engagement of NF-κB and Ku autoantigen (Ku) activation in development of multidrug resistance (MDR) and circumvention of MDR by modulation of NF-κB and Ku. The NF-κB activity and NF-κB p65 subunit level were constitutively higher in MDR cells than in drug-sensitive parental cells. Interestingly, a faster running NF-κB DNA binding complex was identified as Ku, a DNA damage sensor and a key double strand break repair protein, and was positively correlated with the NF-κB activity in MDR cells and Ku- or both subunits of NF-κB-transfected cells. Also both NF-κB and Ku activities were activated or inhibited by treatment with etoposide (VP-16) or MG-132 (a proteasome inhibitor), respectively. Furthermore, PKA inhibitor suppressed markedly the constitutive and drug-induced activities of NF-κB and Ku in MDR cells and subsequently potentiated the cytotoxic activity of anticancer drugs. Our results proposed that the NF-κB and Ku activation could be one of multi-factorial MDR mechanism, and PKA inhibitor, likely via inhibition of NF-κB and Ku activities, could enhance the effectiveness of anticancer drugs against MDR cells with high activities of NF-κB and Ku. *Oncogene* (2001) **20,** 6048–6056.

Keywords: multidrug resistance; NF-κB; Ku autoantigen; PKA inhibitor; anticancer drug

Introduction

Most cancer therapeutics function by killing cells through the induction of the apoptotic pathway. Inherent or acquired resistance to multiple anticancer drugs is one of the major problems in cancer chemotherapy (Gottesman and Pastan, 1993). A major resistance is multidrug resistance (MDR), which is the protection of a cancer cell population against a variety of drugs with different structure and function. Various molecular mechanisms have been associated with MDR in experimental and clinical tumors (Stavrovskaya, 2000). Cancer cells could get MDR phenotype via dysregulation of apoptotic cell death pathways (Minn *et al*., 1995), and resistance to apoptotic stimuli is a principal mechanism by which cancer cells protect against cell killing (Fisher, 1994).

NF-κB has been implicated in apoptosis. NF-κB plays either an anti-apoptotic or a pro-apoptotic role, depending on both the cell type and the nature of the apoptosis-inducing stimuli (Beg and Baltimore, 1996; Bentires-Alj *et al*., 1999). Recent reports have demonstrated the critical role of the NF-κB in protecting cells from apoptosis induced by various anticancer drugs (Mayo and Baldwin, 2000; Wang *et al*., 1996, 1999a) and have suggested that the apoptotic response to conventional chemotherapy may be augmented by the inhibition of NF-κB activation in drug-resistant cancer cells (Cusack *et al*., 2000; Zhou and Kuo, 1997).

Recent evidence revealed that activation of DNA-PK is associated with drug resistance (Christodoulopoulos *et al*., 1998; Muller *et al*., 1998; Shen *et al*., 1998). DNA-PK, a crucial component in recognition and repair of DNA damage, is a nuclear DNA-binding complex composed of a heterodimeric DNA-binding subunit of 70 kDa (Ku70) and 80 kDa (Ku80), and a 460-kDa DNA-PK catalytic subunit (DNA-PKcs) (Jeggo, 1997). Defects in DNA-PK subunits have been shown to result in a reduced capacity to repair DNA double strand breaks (DSBs) and consequent hypersensitivity to radiation (Gu *et al*., 1997; Lees-Miller *et al*., 1995) and etoposide, a DNA-damaging agent (Jin *et al*., 1998). The Ku heterodimer binds to DSBs and appears to stabilize binding of the DNA-PK catalytic subunit to DNA (Blier *et al*., 1993; Jin *et al*., 1997; Smith and Jackson, 1999; Taccioli *et al*., 1994). Once bound to DNA lesions, this complex stimulates DNA repair and signals damage stress responses, triggering the transcriptional activation of p53 (Woo *et al*., 1998) and/or NF-κB (Liu *et al*., 1998), which affects cell cycle arrest, apoptosis, and carcinogenesis. The suppression of DNA-PK activity during the initiation and execution phase of apoptosis will lead to augmentation of apoptotic process (Jhappan *et al*., 2000; Pernin *et al*., 2000; Yang *et al*., 2000). Thus, DNA-PK activity could

*Correspondence: SH Kim; E-mail: ksh7738@hyowon.pusan.ac.kr
[4]These authors equally contributed to this work
Received 15 February 2001; revised 5 June 2001; accepted 14 June 2001

be a determinant in the cellular response to anticancer drugs. Indeed, our previous studies showed that Ku-deficient cells are sensitive to anticancer drugs including non DNA-damaging agents (Kim *et al*., 1999) and an increased expression of Ku participates in the development of MDR (Kim *et al*., 2000).

In mammalian cells, the cAMP-dependent protein kinase (PKA) signal pathway regulates cell proliferation, differentiation and apoptosis (Albert, 1995; Cho-Chung, 1990; Ruchaud *et al*., 1997). NF-κB has been known to be a potential target for PKA. Phosphorylation of p65 subunit of NF-κB by PKA is involved in the activation of NF-κB and stimulates NF-κB-dependent gene expression (Ghosh, 1999; Zhong *et al*., 1997). Therefore, inhibition of PKA may enhance a sensitivity of drug resistant cancer cells against chemotherapeutic agents via down-regulation of NF-κB.

Here, we report that a constitutive activation of NF-κB and Ku could contribute to the acquired MDR phenotype, and inhibition of NF-κB and Ku activities of MDR cells by PKA inhibitors led to an increased cytotoxicity of anticancer drugs and consequently overcame the drug-resistance of MDR cells.

Results

MDR cells showed constitutive activation of NF-κB and Ku

Activation of NF-κB is associated predominantly with protecting cells from apoptosis in certain cell types, and inhibition of NF-κB could trigger apoptosis in cancer cells or sensitize them to cytotoxic drug-induced cell death (Cusack *et al*., 2000; Kasibhatla *et al*., 1998). Therefore, there is a possibility that NF-κB activation could be responsible for the development of MDR. To test this possibility, NF-κB activity and level of nuclear NF-κB p65 subunit in MDR cells were analysed by EMSA and immunoblotting, respectively (Figure 1). The multidrug-resistant FM3A/M cells expressing P-glycoprotein (P-gp) encoded by *MDR1* gene (Figure 1b, bottom panel) showed significantly enhanced constitutive NF-κB activity (Figure 1a) followed by higher basal level of p65 (Figure 1b, top panel) and lower basal level of IκB-α (Figure 1b, middle panel) compared with drug-sensitive parental FM3A cells. Similar results were observed in another MDR cell line, CEM/VLB_{100} cells expressing P-gp (Figure 1b, bottom panel). These results suggest that constitutive activation of NF-κB may be involved in development of MDR.

During the investigation of NF-κB activity in these MDR cells, other DNA-protein complex (complex I), which moved faster than NF-κB and showed higher constitutive DNA binding activity in MDR cells than in their parental cells, was observed. It has been reported that Ku can bind to NF-κB consensus sequence in spinal cord nuclear extract from rabbit (Shackelford *et al*., 1999). Therefore, to test whether Ku contributed to the unidentified complex I,

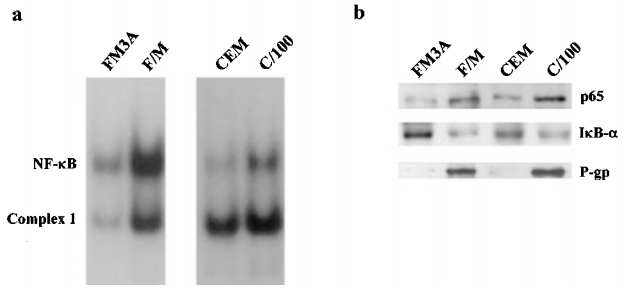

Figure 1 Constitutive NF-κB DNA binding activity and increased level of nuclear p65 subunit in MDR cells. (**a**) The nuclear extracts were isolated from FM3A/M (F/M) and CEM/VLB_{100} (C/100) cells and their respective parental FM3A and CEM cells, and the extracts were analysed for DNA binding activity by EMSA using the ^{32}P-labeled NF-κB consensus oligonucleotide. (**b**) Nuclear NF-κB p65 subunit levels (top panel), cytoplasmic IκBα (middle panel) and P-glycoprotein (P-gp) (bottom panel) of the MDR and their parental cells were evaluated by immunoblot analysis

antibodies to the Ku70 and Ku80 subunits of DNA-PK were used in supershift assays. The complex I of FM3A/M and CEM/VLB_{100} cells was supershifted by both anti-Ku70 and anti-80 antibodies (Figure 2a, right panel), while the upper band was supershifted in the presence of anti-NF-κB p50 antibody (Figure 2a, left panel). To further confirm that Ku is one of the NF-κB binding complexes, EMSA for NF-κB DNA binding activity was performed using Ku-deficient $Ku70^{-/-}$ cells (Figure 2b). $Ku70^{-/-}$ cells did not have the complex I bound to NF-κB consensus oligonucleotide and had a lower NF-κB DNA binding activity compared with their parental cells, and the complex I detected in the parental MEF cells was supershifted by antibody to the Ku70 or Ku80 subunit of DNA-PK. Since dimerization of Ku70 and Ku80 is required for DNA binding, it is likely that the complex I on EMSA represents Ku70/80 dimer complex. Therefore, it could be suggested that the complex I is ascribed to a Ku-DNA complex, and NF-κB DNA binding activity might be correlated with Ku DNA binding activity.

Correlation of NF-κB activity with Ku activity

To probe the association of NF-κB activity with Ku activity, the NF-κB- and Ku-DNA binding activities were assessed by EMSA after treatment of CEM/VLB_{100} cells with etoposide (VP-16), a topoisomerase II inhibitor, known to induce activation of NF-κB (Wang *et al*., 1999b; Watanabe *et al*., 2000), or a proteasome inhibitor (MG-132) known to inhibit NF-κB activity (Pajonk *et al*., 2000). Exposure of the MDR cells to VP-16 ($10 \sim 25$ μM) resulted in concurrent increase of both NF-κB- and Ku-DNA binding activities. The reduction of both activities at a high concentration (50 μM) of VP-16 might be due to the cytotoxic effect of the drug (Figure 3a, top panel). Additionally, we observed a similar pattern of the levels of p65 and Ku proteins (Figure 3a, middle and

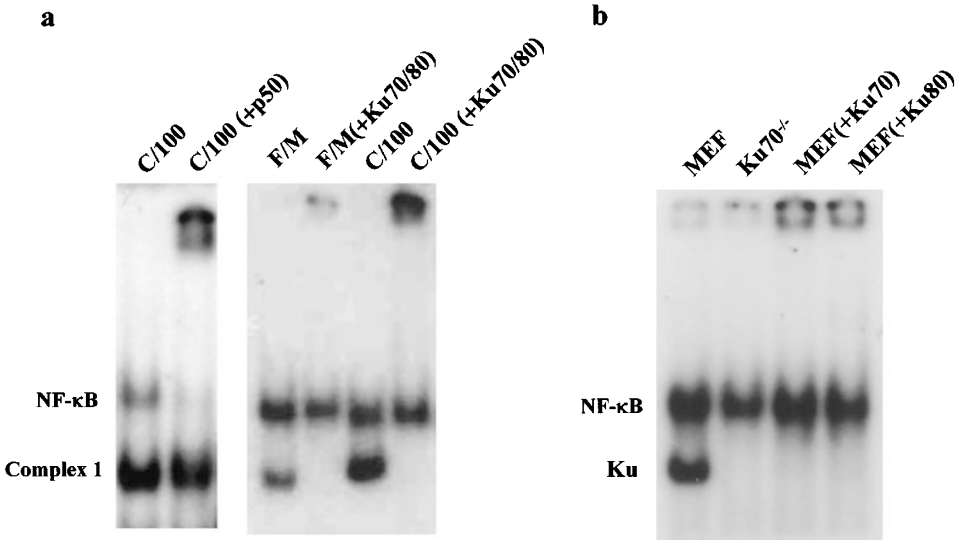

Figure 2 Identification of the Ku as a NF-κB binding complex. The nuclear extracts from MDR cells including FM3A/M (F/M) and CEM/VLB_{100} (C/100) cells (**a**), and MEF and its Ku70-deficient $Ku70^{-/-}$ cells (**b**) were prepared, and were analysed for DNA binding activity by EMSA using the ^{32}P-labeled NF-κB consensus oligonucleotide. For supershift assay, the nuclear extracts were preincubated with anti-p50 antibody (**a**, left panel) or mixture (Ku70/80) of anti-Ku70 and anti-Ku80 antibodies (**a**, right panel) for MDR cells, or anti-Ku70 or anti-Ku80 antibody for MEF cells (**b**) and subjected to EMSA

bottom panels). By contrast, treatment of the MDR cells with MG-132 resulted in concurrent decrease of NF-κB- and Ku-DNA binding activities in a dose-dependent manner (Figure 3b, top panel), and these were followed by the decrease of p65 and Ku proteins of the MDR cells (Figure 3b, middle and bottom panels). These results suggest that NF-κB activity may be correlated with Ku activity. To confirm this possibility, NF-κB and Ku activities were examined in R7080-6 cells overexpressing both human Ku70 and Ku80 (Kim *et al*., 2000) and PC12-NF-κB cells overexpressing both subunits of NF-κB (Lee *et al*., 2001). R7080-6 cells showed markedly increased NF-κB activity as well as Ku activity and a higher p65 level compared with parental Rat-1 cells (Figure 4a). In addition, PC12-NF-κB cells with constitutive NF-κB activity exhibited a significantly increased Ku activity and a higher cellular Ku level compared with parental PC12 cells (Figure 4b). These results provided strong evidence for the correlation between NF-κB and Ku activity.

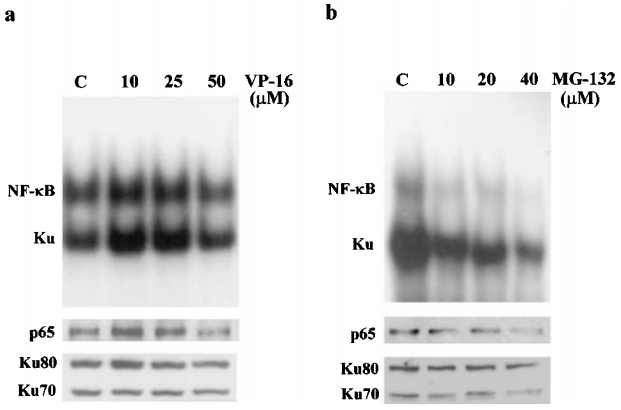

Figure 3 Modulation of NF-κB and Ku activities by etoposide or MG-132. CEM/VLB_{100} cells were exposed to the indicated doses of etoposide (VP-16) for 2 h (**a**) or MG-132 for 6 h (**b**), and the nuclear extracts were prepared and subjected to EMSA (top panels). Immunoblot analyses were done to measure the NF-κB and Ku expression levels of VP-16 or MG-132-treated cells (middle and bottom panels)

Inhibition of the constitutive and drug-induced NF-κB and Ku activities in MDR cells by PKA inhibitors

The transcriptional activity of NF-κB can be regulated through phosphorylation of NF-κB by PKA (Zhong *et al*., 1997). Therefore, it was examined whether PKA inhibitor can inhibit both constitutive NF-κB and Ku activities in MDR cells. CEM/VLB_{100} cells were treated with the PKA inhibitor, H-87 (2∼25 μM) or KT5720 (5∼500 nM) for 6 h. Exposure of CEM/VLB_{100} cells to H-87 caused concurrent decrease in both NF-κB and Ku activities (Figure 5a, top panel) and these were followed by decrease of p65 and Ku levels in a dose-dependent manner (Figure 5a, middle and bottom panels). Similar results were obtained when cells were treated with KT5720 (Figure 5b). These results indicated that the inhibition of PKA could affect both activities of NF-κB and Ku simultaneously. In addition, we examined the effect of PKA inhibitor on anticancer drug-induced NF-κB and Ku activation in MDR cells. When CEM/VLB_{100} cells were treated with various doses of vincristine (VCR) in the presence or absence of 100 nM KT5720 for 6 h, VCR-induced NF-κB and Ku activation was significantly prevented by KT5720 treatment (Figure 6a, top panel) and these were followed by decrease of p65 and Ku levels (Figure 6a, middle and bottom panels). Also, when the MDR cells were treated with various doses of

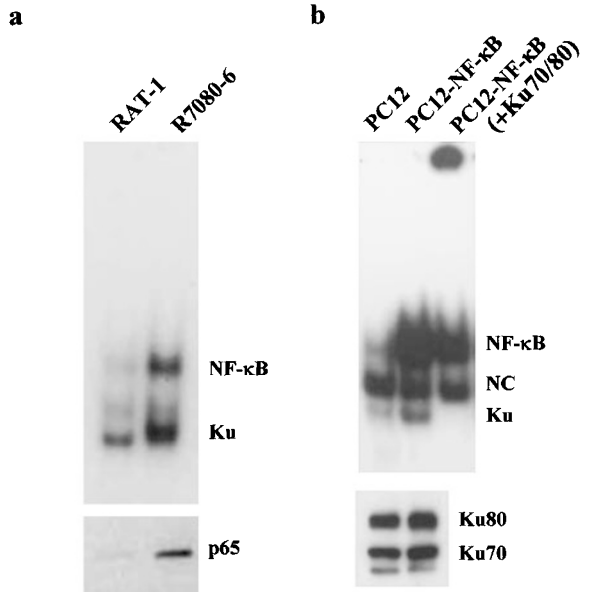

Figure 4 NF-κB and Ku activation in Ku- or NF-κB-overexpressing cells. Nuclear extracts isolated from RAT-1 and its Ku70/80-overexpressing R7080-6 cells (**a**) or PC12 and its NF-κB-overexpressing PC12-NF-κB cells (**b**) were assayed for DNA binding activity by EMSA using the ^{32}P-labeled NF-κB consensus oligonucleotide. For supershift assay, equal amount of nuclear extracts of PC12-NF-κB cells was incubated with mixture of anti-Ku70/80 (Ku) antibodies before the binding reaction and was analysed by EMSA. The retarded band labeled NC represents nonspecific binding complex (upper panels). Immunoblot analysis was done to measure p65 level in RAT-1 and its Ku70/80-overexpressing R7080-6 cells or Ku level in PC12 and its NF-κB-overexpressing PC12-NF-κB cells (lower panels)

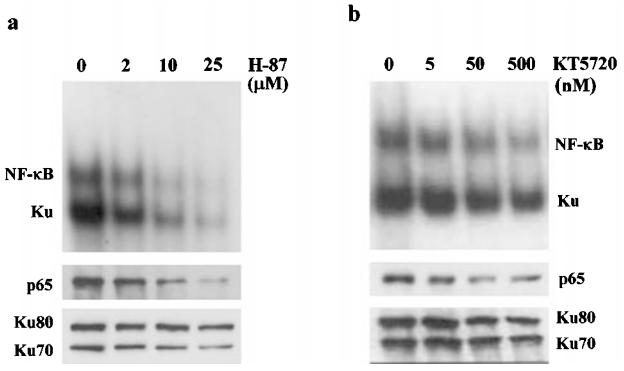

Figure 5 Inhibition of NF-κB and Ku activities by protein kinase A inhibitors. CEM/VLB$_{100}$ cells were exposed to the indicated doses of H-87 (**a**) or KT5720 (**b**) for 6 h, and the NF-κB and Ku DNA binding activities were measured by EMSA (top panels). Immunoblot analysis was done to measure Ku and p65 expression levels of protein kinase A inhibitor-treated cells (middle and bottom panels)

VP-16 in the presence or absence of 100 nM KT5720 for 3 h, VP-16-induced NF-κB and Ku activation was markedly inhibited by KT5720 treatment (Figure 6b). These results demonstrated that anticancer drug-induced NF-κB and Ku activation in MDR cells could be prevented by treatment of PKA inhibitors.

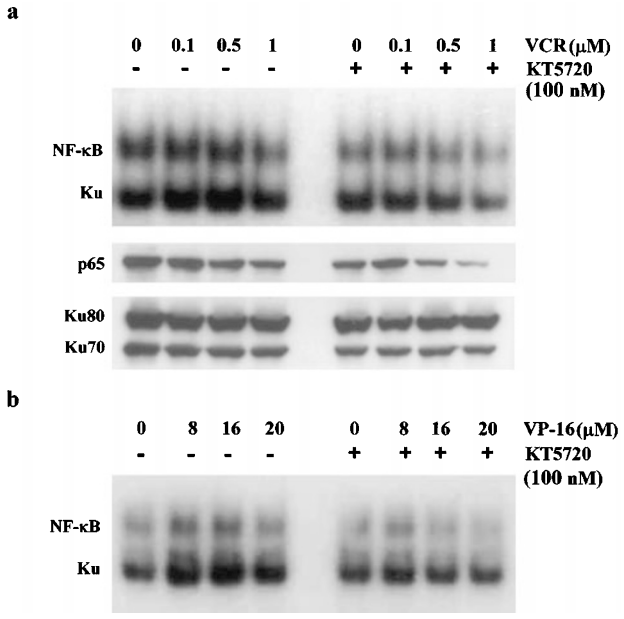

Figure 6 Effect of PKA inhibitor on anticancer drug-induced NF-κB and Ku activities in MDR cells. CEM/VLB$_{100}$ cells were treated with the indicated doses of vincristine in the presence or absence of 100 nM KT5720 for 6 h, and EMSA and immunoblot analysis were done (**a**). Also, the cells were treated with the indicated doses of etoposide (VP-16) in the presence or absence of 100 nM KT5720 for 3 h, and EMSA was done (**b**)

PKA inhibitors markedly potentiated cytotoxicity of anticancer drugs to MDR cells

To determine whether inhibition of NF-κB and Ku activities by PKA inhibitors leads to enhancement of cytotoxic response of MDR cells against anticancer drugs, and thereby reverse the drug resistance of MDR cells, MDR and their parental cells were treated with various concentrations of anticancer drugs in the presence or absence of PKA inhibitors (H-87, H-89 and KT5720) for 96 h, and cytotoxicity was determined by MTT assay. The results are summarized in Tables 1 and 2. The degree of drug sensitization by PKA inhibitors was expressed as the ratio of the IC_{50} for anticancer drug alone to the IC_{50} for combined treatment of anticancer drug and PKA inhibitor. H-87 significantly potentiated the cytotoxic effects of vincristine (VCR), vinblastine (VLB), adriamycin (ADR) and VP-16 on CEM/VLB$_{100}$ cells (Table 1). Treatment of 4 μM H-87 sensitized CEM/VLB$_{100}$ cells approximately 94-, 77-, 15- and sixfold to VCR, VBL, ADR and VP-16, respectively, and therefore resulted in restoring drug sensitivity of MDR cells. Another isoquinoline sulfonamide compound, H-89, also significantly potentiated the cytotoxicity of these P-gp-related drugs but lower degree than H-87. In the CEM/VLB$_{100}$ cells, KT5720 also showed the high combination effect with anticancer drugs at a concentration of 10 nM. By contrast, parental CEM cells were much less responsive to the combined effect of anticancer drugs and PKA inhibitor under the same conditions used for

Table 1 Potentiation of chemosensitivity by PKA inhibitors in CEM and CEM/VLB_{100} cells

Cells	*Drug*	*Inhibitor free*	IC_{50} *+4 μM H-87*	*+5 μM H-89*	*+10 nM KT5720*
CEM	VCR (nM)	0.4	0.2 (2.0)	0.4 (1.0)	0.3 (1.3)
	VLB (nM)	3.9	3.3 (1.2)	3.5 (1.1)	3.6 (1.1)
	ADR (ng/ml)	5.6	3.1 (1.8)	3.9 (1.4)	2.1 (2.7)
	VP-16 (nM)	205.3	186.6 (1.1)	114.1 (1.8)	205.3 (1.0)
CEM/VLB_{100}	VCR (nM)	178.6	1.9 (94.0)	4.6 (38.8)	14.9 (12.0)
	VLB (nM)	269.5	3.5 (77.0)	7.8 (34.6)	61.4 (4.4)
	ADR (ng/ml)	160.0	11.0 (14.6)	21.0 (7.6)	29.1 (5.5)
	VP-16 (nM)	957.7	157.0 (6.1)	195.4 (4.9)	319.2 (3.0)

Each cell line (2×10^4 cells/ml) was treated with each anticancer drug for 96 h in the presence or absence of PKA inhibitor (H-87, H-89 or KT5720). Growth inhibition assay was performed by MTT method. Values in parentheses indicate the ratio of IC_{50} for drug alone to the IC_{50} for drug in the presence of PKA inhibitor. Values are the average of (at least) two independent experiments and triplicate determinants in each experiment. Vincristine, VCR; Vinblistine, VLB; Adriamycin, ADR; Etoposide, VP-16

Table 2 Potentiation of chemosensitivity by PKA inhibitors in FM3A and FM3A/M cells

Cells	*Drug*	*Inhibitor free*	IC_{50} *+4 μM H-87*	*+5 μM H-89*	*+10 nM KT5720*
FM3A	VCR (nM)	4.7	3.9 (1.2)	3.6 (1.3)	4.7 (1.0)
	VLB (nM)	6.8	6.8 (1.0)	5.2 (1.3)	6.8 (1.0)
	ADR (ng/ml)	37.0	16.8 (2.2)	28.5 (1.3)	33.6 (1.1)
FM3A/M	VCR (nM)	75.7	8.9 (8.5)	10.0 (7.6)	31.7 (2.4)
	VLB (nM)	74.0	8.5 (8.7)	8.4 (8.8)	14.8 (5.0)
	ADR (ng/ml)	150.3	28.9 (5.2)	36.6 (4.1)	34.1 (4.4)

Each cell line (2×10^4 cells/ml) was treated with each anticancer drug for 96 h in the presence or absence of PKA inhibitor (H-87, H-89 or KT5720). Values in parentheses indicate the ratio of IC_{50} for drug alone to the IC_{50} for drug in the presence of PKA inhibitor. Values are the average of (at least) two independent experiments and triplicate determinants in each experiment. Vincristine, VCR; Vinblistine, VLB; Adriamycin, ADR

CEM/VLB_{100} cells. Similarly, significant combination effect of anticancer drugs and PKA inhibitor was shown in FM3A/M cells (Table 2). In addition, we have examined the combination effect of PKA inhibitor on the cytotoxicity of anticancer drug using PC12-NF-κB and $Ku70^{-/-}$ cells and their parental cells to show the relationship between activities of NF-κB and Ku and drug resistance (Table 3). PC12-NF-κB cells were less sensitive to anticancer drugs than PC12 cells, but the NF-κB overexpressing cells were more responsive to the combination of PKA inhibitor and anticancer drug than PC12 cells. Treatment of 5 μM H-89 sensitized PC12-NF-κB cells approximately 20-, 13- and fourfold to VCR, VP-16 and ADR, respectively, while exposure of PC12 cells to anticancer drugs and H-89 resulted in only a small increase in cytotoxicity of the drugs. On the other hand, $Ku70^{-/-}$ cells were more sensitive to anticancer drugs than parental MEF cells but chemosensitization effect of PKA inhibitor decreased significantly compared with MEF cells. Treatment of 5 μM H-89 increased chemosensitivity of MEF cells against VCR, VP-16 and ADR to approximately six-, nine- and eightfold, respectively, but the chemosensitization effect of H-89 was markedly decreased in $Ku70^{-/-}$ cells to approximately 1.3-, 1.2- and fivefold, respectively. These results suggest that the chemosensitization effect of PKA inhibitor may be attributed to cellular NF-κB and Ku activities.

On the other hand, PC12-NF-κB cells and R7080-6 cells had an increased level of P-gp, although these cells were not selected against anticancer drugs (Figure 7), suggesting that expression of P-gp can be regulated directly or indirectly by NF-κB-dependent pathway. Therefore, there is a possibility that PKA inhibitors enhance the effect of cytotoxic drugs against MDR cells at least in part by down-regulation of *MDR1* expression, since *MDR1* gene expression has been known to be suppressed by inhibition of PKA (Kim *et al*., 1993).

Discussion

It has been proposed that potential apoptotic stimuli initiate two distinct signaling pathways, one that leads to activation of apoptosis and one that leads to NF-κB activation, which induces a cell survival response through the inhibition of apoptosis (Kaltschmidt *et al*., 2000). Some chemotherapeutic agents activate NF-κB, and this leads to suppression of the apoptotic potential of the chemotherapy (Wang *et al*., 1996). Recent studies have proposed that activation of NF-κB in response to chemotherapy could be a principal mechanism of transiently inducible chemoresistance which protects cancer cells from acute stress (Cusack *et al*., 2000; Wang *et al*., 1999a). Here, we have studied whether NF-κB is associated with a constitutive MDR phenotype.

Table 3 Potentiation of chemosensitivity by H-89 in PC12-NF-κB and Ku70$^{-/-}$ cells and their parental cells

Cells	*Drugs*	IC_{50} *Inhibitor free*	IC_{50} *+5 μM H-89*
PC12	VCR (nM)	70.2	13.0 (5.4)
	VP-16 (μM)	0.6	0.2 (3.0)
	ADR (ng/ml)	37.4	34.0 (1.1)
PC12-NF-κB	VCR (nM)	495.0	25.0 (19.8)
	VP-16 (μM)	5.2	0.4 (13.0)
	ADR (ng/ml)	140.6	37.0 (3.8)
MEF	VCR (nM)	17.1	3.1 (5.5)
	VP-16 (nM)	67.9	7.9 (8.6)
	ADR (ng/ml)	18.0	2.2 (8.2)
Ku70$^{-/-}$	VCR (nM)	3.0	2.3 (1.3)
	VP-16 (nM)	17.9	14.9 (1.2)
	ADR (ng/ml)	2.0	0.4 (5.0)

Each cell line (2×10^4 cells/ml) was treated with each anticancer drug for 96 h in the presence or absence of H-89. Values in parentheses indicate the ratio of IC_{50} for drug alone to the IC_{50} for drug in the presence of H-89. Values are the average of (at least) two independent experiments and triplicate determinants in each experiment. Vincristine, VCR; Etoposide, VP-16; Adriamycin, ADR

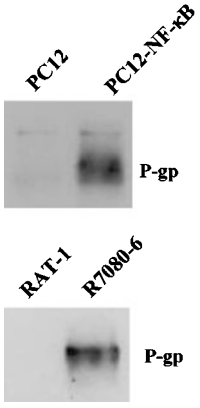

Figure 7 Level of P-glycoprotein (P-gp) in Ku- or NF-κB-overexpressing cells. Plasma membrane proteins were separated on 5% SDS–PAGE and probed with anti-P-gp antibody to measure P-gp expression levels of Ku- or NF-κB-overexpressing cells

In the present study, MDR cells with a high level of P-gp exhibited markedly increased constitutive NF-κB DNA binding activity and p65 subunit level of NF-κB compared with drug-sensitive parental cells. It is now increasingly evident that MDR phenotype may result from multiple factors even in the same type of cancer (Baldini, 1997; McKenna and Padua, 1997). For example, there are some reports showing that P-gp-associated MDR cells develop other mechanisms of drug resistance such as increase of glutathione level, decrease of growth rate and topoisomerase II, overexpression of MRP (multidrug resistance-associated protein), overexpression of Bcl-2 antiapoptotic protein (Chao, 1996; Mickisch *et al*., 1991; Neumann *et al*., 1992; Palissot *et al*., 1998; Satta *et al*., 1992; Zhou *et al*., 1996), and down-regulation of JNK/SAPK (Kang *et al*., 2000). Therefore, NF-κB might be a new additional mechanism of drug resistance in MDR cells with high level of P-gp.

During the investigation of NF-κB activity in MDR cells, we observed that other DNA-protein complex moving faster than NF-κB was Ku, a DNA damage sensor and a key double strand break repair protein. This finding is consistent with that reported recently by Shackelford *et al*. (1999), in which a complex binding to an oligonucleotide containing the NF-κB motif was identified as Ku in rabbit spinal cord model of reversible ischemia. In the present study, we showed that both NF-κB and Ku DNA binding activities were constitutively activated in MDR cells and appeared to be positively correlated with each other as shown in etoposide or MG-132-treated cells and the NF-κB- or Ku-overexpressing cells. However, we do not know why both NF-κB and Ku activities were constitutively up-regulated in MDR cells and how NF-κB or Ku activity regulate each other activity mutually. Since NF-κB and Ku can be activated in response to stresses including anticancer drugs, and play an antiapoptotic role, there is a possibility that MDR cells with high activities of NF-κB and Ku might be selected during chronic exposure to cytotoxic drugs. On the other hand, since NF-κB functions as a transcription factor, activation of NF-κB may lead to the induction of Ku directly or indirectly. Recently, it has been reported that DNA-PKcs participate in the activation of NF-κB following by exposure to DNA damage agent (Basu *et al*., 1998). Then the increased Ku activity will lead to activation of DNA-PK and consequently activation of NF-κB. In the present experiment, we provided for the first time strong evidence for the association of constitutive NF-κB and Ku activities with MDR phenotype and the correlation between NF-κB and Ku activities.

Ku, the DNA binding component of DNA-PK, plays a central role in the recognition of DNA damage since it binds to DSBs due to ionizing radiation or exposure of anticancer drug (Bjork-Eriksson *et al*., 1999; Muller *et al*., 1998; Zhao *et al*., 2000), and initiates the DNA repair process (Wang *et al*., 1998). Previously, we have shown that Ku can affect chemosensitivity (Kim *et al*., 1999), and an increase in Ku expression is associated with MDR phenotype (Kim *et al*., 2000). There are some evidences that an enhanced expression of DNA-PK could contribute to development of drug resistance. Tumor cells resistant to adriamycin showed the increase in DNA-PK expression (Shen *et al*., 1998). Also, an increase in DNA-PK activity correlated with chlorambucil resistance (Christodoulopoulos *et al*., 1998; Muller *et al*., 1998), or contributed to development of MDR (Kim *et al*., 2000). In addition, it has been reported that DNA-PK protects against heat-induced apoptosis (Nueda *et al*., 1999). Therefore, constitutive NF-κB and Ku activities might contribute in part to additional mechanism of MDR phenotype in P-gp-overexpressing MDR cells.

Although there are several pathways leading to activation of NF-κB (DiDonato *et al*., 1997), PKA also play a role in regulation of NF-κB activity. It has been shown that PKA-mediated phosphorylation of

NF-κB leads to increase of the transcriptional activity (Zhong *et al*., 1997). In our study, constitutive and drug-stimulated activities of NF-κB and Ku in MDR cells were inhibited by PKA inhibitors, supporting that NF-κB can be activated by PKA, and PKA inhibitors markedly potentiated cytotoxicity of anticancer drugs against MDR cells compared with their parental cells. Since MDR cells used in this experiment express high level of *MDR1* gene, and PKA inhibitor has been known to inhibit the expression of *MDR1* gene (Cvijic and Chin, 1997; Pae *et al*., 1999), these results could be in part due to down-regulation of *MDR1* gene by PKA inhibitors. Although we showed an increased and decreased chemosensitization in NF-κB-overexpressed PC12-NF-κB cells and Ku-deficient Ku70$^{-/-}$ cells by PKA inhibitor, respectively, it was impossible to rule out the possibility that down-regulation of P-gp is involved in enhanced chemosensitivity by inhibition of PKA in MDR cells with constitutive activities of NF-κB and Ku. In fact, PC12-NF-κB cells and R7080-6 cells had a higher level of P-gp than that of their parental cells. However, we have shown previously that R7080-6 cells were 3.6-fold more resistant to bleomycin, a P-gp-unrelated drug, than their parental Rat-1 cells (Kim *et al*., 1999) and there are several antiapoptotic genes such as TRAF1 and 2 and c-IAP1 and 2, the Bcl-2 homologs A1/Bfl-1 and Bcl-xL, IEX-1, and XIAP, which can be transcriptionally activated by NF-κB (Baldwin, 2001; Yamamoto and Gaynor, 2001). Therefore, although the enhanced chemosensitivity by inhibition of PKA in MDR cells with constitutive activities of NF-κB and Ku could be in part due to down-regulation of P-gp, transcription of which appears to be directly or indirectly regulated by NF-κB, chemoresistance of MDR cells with constitutive activities of NF-κB and Ku would be restored by PKA inhibitor via suppression of NF-κB and Ku activities and subsequent decrease of anti-apoptotic target genes.

In conclusion, the results presented in this study shows evidence that drug resistance of MDR cells could be correlated with the constitutive activities of NF-κB and Ku, which are known to prevent apoptosis, and it may contribute to a new mechanism of multifactorial MDR phenotype. At present, we do not know much about the interrelated regulation between NF-κB and Ku activities, and thus further experiments will be required to clarify the precise mechanisms of correlation of NF-κB and Ku.

Materials and methods

Materials

The following reagents were obtained from the listed sources and used at the concentrations indicated in the text. N-[2-(p-bromo cinnamyl amino)ethyl]-5-isoquinoline sulfonamide (H-89) and KT5720 were purchased from Calbiochem (San Diego, CA, USA). N-[2-(p-bromo cinnamyl methylamino)ethyl]-5-isoquinoline sulfonamide (H-87) was kindly donated by Dr Hidaka (University of Nagoya, Japan). Vinblastine, vincristine, adriamycin and etoposide were obtained from Sigma. All other materials were obtained in the highest grade. The following antibodies were used in these studies. Rabbit anti-Ku70 and anti-Ku80 antibodies were provided by Dr Li (Memorial Sloan-Kettering Cancer Center, NY, USA). The rabbit anti-c-Rel (p65) polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. Secondary antibody was obtained from Boehringer Mannheim.

Cell lines and culture conditions

CEM human leukemia cells and its MDR subline, vinblastine (VLB)-resistant CEM/VLB$_{100}$ expressing high level of *MDR1* genes (kindly donated by Dr Beck, University of Illinois) were cultured in RPMI supplemented with 10% fetal bovine serum (FBS, GIBCO BRL, Life Technologies, Inc.), 100 units/ml penicillin, and 100 μg/ml streptomycin. Multidrug-resistant FM3A/M cells were isolated from the FM3A mouse mammary carcinoma cells by stepwise treatment of colchicine, and exhibited MDR to vinblastine, adriamycin and actinomycin D, as described previously (Kim *et al*., 2000). Both FM3A and FM3A/M cells also were cultured in RPMI complete medium. Murine embryonic fibroblast MEF and its Ku deficient Ku70$^{-/-}$ cell lines presented above were kindly donated by Dr Li (Memorial Sloan-Kettering Cancer Center, NY, USA).

Cell proliferation assay

Cells were seeded in 96-well plates at 2×10^4 cells/ml and treated with each anticancer drug in the presence or absence of each PKA inhibitor for 96 h, followed by the addition of 100 μl of MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] solution (5 mg/ml), and incubation for 4 h in the dark room. The water-insoluble MTT-formazan crystals were dissolved in dimethyl sulfoxide, and reduction of MTT was determined at 570 nm using ELISA reader (Bio-Tec Instruments). The concentration of each anticancer drug which reduced cell growth by 50% after 96 h treatment (IC_{50}) was determined from the growth inhibition plots.

Electrophoretic mobility shift analysis (EMSA)

Cells (1×10^6 cells/well) were exposed to the indicated doses of drug in the presence or absence of PKA inhibitor for indicated periods, and preparation of nuclear extract and electrophoretic mobility shift analysis (EMSA) was performed as described previously (Kim *et al*., 1999, 2000). The extracted nuclear proteins (20 μg (for CEM, CEM/VLB$_{100}$ cells and HeLa cells) or 50 μg (for FM3A, FM3A/M cells and DNA-PK-related cell lines)) were incubated with ^{32}P-labeled NF-κB consensus oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′). The protein-bound and free oligonucleotides were electrophoretically separated on 4.5% native polyacrylamide gels in 0.5×TBE buffer (44.5 mM Tris (pH 8.0), 44.5 mM boric acid and 1 mM EDTA) for 3 h at 120 V. The gels were dried and autoradiographed. For gel mobility supershift assay, 1 μg of monoclonal antibody specific to Ku70, Ku80 or p50 was incubated with nuclear extracts for 20 min at 21°C prior to the Ku or NF-κB DNA binding reaction, respectively.

Immunoblot analysis

Plasma membrane proteins (200 μg) isolated from FM3A/M and CEM/VLB$_{100}$ cells and their respective parental FM3A

and CEM cells were separated on 5% SDS-polyacrylamide gel or the cell lysates (whole cell or nuclear) containing an equal amount of protein (50 μg) were resolved on a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were blotted to Hybond ECL nitrocellulose membrane (Amersham Life Science, Arlington Heights, IL, USA). The membrane was incubated with antibodies specific to P-glycoprotein (P-gp), NF-κB p65 subunit or Ku70/80 (anti-P-gp from Calbiochem, Inc., anti-NF-κB p65 or p50 from Santa cruiz Biotechnology and anti-Ku70/80 from Dr Li of Memorial Sloan-Kettering Cancer Center), followed by secondary antibody conjugated with horseradish peroxidase. Immunoreactivity was detected by the enhanced chemiluminescent (ECL) detection using the ECL Western blotting detection reagent (Pierce, USA).

Acknowledgments
This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea. (HMP-00-B-20900-0089)

References

Albert DA. (1995). *J. Clin. Invest.,* **95,** 1490 – 1496.
Baldini N. (1997). *Nat. Med.,* **3,** 378 – 380.
Baldwin AS. (2001). *J. Clin. Invest.,* **107,** 241 – 246.
Basu S, Rosenzweig KR, Youmell M and Price BD. (1998). *Biochem. Biophys. Res. Commun.,* **247,** 79 – 83.
Beg AA and Baltimore D. (1996). *Science,* **274,** 782 – 784.
Bentires-Alj M, Hellin AC, Ameyar M, Chouaib S, Merville MP and Bours V. (1999). *Cancer Res.,* **59,** 811 – 815.
Bjork-Eriksson T, West C, Nilsson A, Magnusson B, Svensson M, Karlsson E, Slevin N, Lewensohn R and Mercke C. (1999). *Int. J. Radiat. Oncol. Biol. Phys.,* **45,** 1005 – 1010.
Blier PR, Griffith AJ, Craft J and Hardin JA. (1993). *J. Biol. Chem.,* **268,** 7594 – 7601.
Chao CC. (1996). *Eur. J. Pharmacol.,* **305,** 213 – 222.
Cho-Chung YS. (1990). *Cancer Res.,* **50,** 7093 – 7100.
Christodoulopoulos G, Muller C, Salles B, Kazmi R and Panasci L. (1998). *Cancer Res.,* **58,** 1789 – 1792.
Cusack Jr JC, Liu R and Baldwin Jr AS. (2000). *Cancer Res.,* **60,** 2323 – 2330.
Cvijic ME and Chin KV. (1997). *Cell Growth Differ.,* **8,** 1243 – 1247.
DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E and Karin M. (1997). *Nature,* **388,** 548 – 554.
Fisher DE. (1994). *Cell,* **78,** 539 – 542.
Ghosh S. (1999). *Immunol. Res.,* **19,** 183 – 189.
Gottesman MM and Pastan I. (1993). *Annu. Rev. Biochem.,* **62,** 385 – 427.
Gu Y, Jin S, Gao Y, Weaver DT and Alt FW. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 8076 – 8081.
Jeggo PA. (1997). *Mutat. Res.,* **384,** 1 – 14.
Jhappan C, Yusufzai TM, Anderson S, Anver MR and Merlino G. (2000). *Mol. Cell. Biol.,* **20,** 4075 – 4083.
Jin S, Inoue S and Weaver DT. (1997). *Cancer Surv.,* **29,** 221 – 261.
Jin S, Inoue S and Weaver DT. (1998). *Carcinogenesis,* **19,** 965 – 971.
Kaltschmidt B, Kaltschmidt C, Hofmann TG, Hehner SP, Droge W and Schmitz ML. (2000). *Eur. J. Biochem.,* **267,** 3828 – 3835.
Kang CD, Ahn BK, Jeong CS, Kim KW, Lee HJ, Yoo SD, Chung BS and Kim SH. (2000). *Exp. Cell. Res.,* **256,** 300 – 307.
Kasibhatla S, Brunner T, Genestier L, Echeverri F, Mahboubi A and Green DR. (1998). *Mol. Cell.,* **1,** 543 – 551.
Kim SH, Kim D, Han JS, Jeong CS, Chung BS, Kang CD and Li GC. (1999). *Cancer Res.,* **59,** 4012 – 4017.
Kim SH, Park JI, Chung BS, Kang CD and Hidaka H. (1993). *Cancer Lett.,* **74,** 37 – 41.
Kim SH, Um JH, Dong-Won B, Kwon BH, Kim DW, Chung BS and Kang CD. (2000). *Leuk. Res.,* **24,** 917 – 925.
Lee HJ, Kim SH, Kim KW, Um JH, Lee HW, Chung BS and Kang CD. (2001). *J. Neurochem.,* **76,** 602 – 609.
Lees-Miller SP, Godbout R, Chan DW, Weinfeld M, Day III RS, Barron GM and Allalunis-Turner J. (1995). *Science,* **267,** 1183 – 1185.
Liu L, Kwak YT, Bex F, Garcia-Martinez LF, Li XH, Meek K, Lane WS and Gaynor RB. (1998). *Mol. Cell. Biol.,* **18,** 4221 – 4234.
Mayo MW and Baldwin AS. (2000). *Biochim. Biophys. Acta,* **1470,** M55 – M62.
McKenna SL and Padua RA. (1997). *Br. J. Haematol.,* **96,** 659 – 674.
Mickisch G, Fajta S, Bier H, Tschada R and Alken P. (1991). *Urol. Res.,* **19,** 99 – 103.
Minn AJ, Rudin CM, Boise LH and Thompson CB. (1995). *Blood,* **86,** 1903 – 1910.
Muller C, Christodoulopoulos G, Salles B and Panasci L. (1998). *Blood,* **92,** 2213 – 2219.
Neumann M, Wilisch A, Diddens H, Probst H and Gekeler V. (1992). *Anticancer Res.,* **12,** 2297 – 2302.
Nueda A, Hudson F, Mivechi NF and Dynan WS. (1999). *J. Biol. Chem.,* **274,** 14988 – 14996.
Pae HO, Yoo JC, Jun CD, Paik SG, Choi BM, Baek KH, Kim JM and Chung HT. (1999). *Immunopharmacol. Immunotoxicol.,* **21,** 233 – 245.
Pajonk F, Pajonk K and McBride WH. (2000). *Int. J. Radiat. Oncol. Biol. Phys.,* **47,** 1025 – 1032.
Palissot V, Belhoussine R, Carpentier Y, Sebille S, Morjani H, Manfait M and Dufer J. (1998). *Biochem. Biophys. Res. Commun.,* **245,** 918 – 922.
Pernin D, Uhrhammer N, Verrelle P, Bignon YJ and Bay JO. (2000). *Bull. Cancer,* **87,** 635 – 641.
Ruchaud S, Seite P, Foulkes NS, Sassone-Corsi P and Lanotte M. (1997). *Oncogene,* **15,** 827 – 836.
Satta T, Isobe K, Yamauchi M, Nakashima I and Takagi H. (1992). *Cancer,* **69,** 941 – 946.
Shackelford DA, Tobaru T, Zhang S and Zivin JA. (1999). *J. Neurosci.,* **19,** 4727 – 4738.
Shen H, Schultz M, Kruh GD and Tew KD. (1998). *Biochim. Biophys. Acta.,* **1381,** 131 – 138.
Smith GC and Jackson SP. (1999). *Genes Dev.,* **13,** 916 – 934.
Stavrovskaya AA. (2000). *Biochemistry (Mosc),* **65,** 95 – 106.
Taccioli GE, Gottlieb TM, Blunt T, Priestley A, Demengeot J, Mizuta R, Lehmann AR, Alt FW, Jackson SP and Jeggo PA. (1994). *Science,* **265,** 1442 – 1445.
Wang CY, Cusack Jr JC, Liu R and Baldwin Jr AS. (1999a). *Nat. Med.,* **5,** 412 – 417.
Wang CY, Guttridge DC, Mayo MW and Baldwin Jr AS. (1999b). *Mol. Cell. Biol.,* **19,** 5923 – 5929.
Wang CY, Mayo MW and Baldwin Jr AS. (1996). *Science,* **274,** 784 – 787.

Wang J, Dong X and Reeves WH. (1998). *J. Biol. Chem.,* **273,** 31068 – 31074.

Watanabe K, Kubota M, Hamahata K, Lin Y and Usami I. (2000). *Biochem. Pharmacol.,* **60,** 823 – 830.

Woo RA, McLure KG, Lees-Miller SP, Rancourt DE and Lee PW. (1998). *Nature,* **394,** 700 – 704.

Yamamoto Y and Gaynor RB. (2001). *J. Clin. Invest.,* **107,** 135 – 142.

Yang CR, Leskov K, Hosley-Eberlein K, Criswell T, Pink JJ, Kinsella TJ and Boothman DA. (2000). *Proc. Natl. Acad. Sci. USA,* **97,** 5907 – 5912.

Zhao HJ, Hosoi Y, Miyachi H, Ishii K, Yoshida M, Nemoto K, Takai Y, Yamada S, Suzuki N and Ono T. (2000). *Clin. Cancer Res.,* **6,** 1073 – 1078.

Zhong H, SuYang H, Erdjument-Bromage H, Tempst P and Ghosh S. (1997). *Cell,* **89,** 413 – 424.

Zhou DC, Ramond S, Viguie F, Faussat AM, Zittoun R and Marie JP. (1996). *Int. J. Cancer,* **65,** 365 – 371.

Zhou G and Kuo MT. (1997). *J. Biol. Chem.,* **272,** 15174 – 15183.