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Activation of the c-Jun NH_2 terminal kinase (JNK) signaling pathway is essential during PBOX-6-induced apoptosis in chronic myelogenous leukemia (CML) cells

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PBOX-6 induces JNK activation in CML cells

ABSTRACT

The mitogen-activated protein (MAP) kinase family is activated in response to a wide variety of external stress signals such as UV irradiation, heat shock and many chemotherapeutic drugs and leads to the induction of apoptosis. A novel series of pyrrolo-1,5-benzoxazepines have been shown to potently induce apoptosis in chronic myelogenous leukemia (CML) cells which are resistant to many chemotherapeutic agents. In this study we delineate part of the mechanism by which a representative compound known as PBOX-6 induces apoptosis. We investigate whether PBOX-6 induces activation of MAP kinase signaling pathways in CML cells. Treatment of K562 cells with PBOX-6 resulted in the transient activation of two JNK isoforms, JNK 1 and JNK2. In contrast, PBOX-6 did not activate the extracellular signal regulated kinase (ERK) or p38. Apoptosis was found to occur independently of the small GTPases Ras, Rac and Cdc42, but involves phosphorylation of the JNK substrates, c-jun and ATF-2. Pretreatment of K562 cells with the JNK inhibitor, dicoumarol, abolished PBOX-6-induced phosphorylation of c-jun and ATF-2, and inhibited the apoptosis induced, suggesting that JNK activation is an essential component of the apoptotic pathway induced by PBOX-6. Consistent with this finding, transfection of K562 cells with the JNK scaffold protein, JIP-1, inhibited JNK activity and apoptosis induced by PBOX-6. JIP-1 specifically scaffolds JNK, MKK7 and members of the mixed-lineage kinase (MLK) family, implicating these kinases upstream of JNK in the apoptotic pathway induced by PBOX-6 in K562 cells.

INTRODUCTION

Mitogen-activated protein (MAP) kinases are a group of protein serine/threonine kinases that are activated in response to a variety of extracellular stimuli and mediate signal transduction cascades that play an important regulatory role in cell growth, differentiation, and apoptosis (1). In mammalian systems, the biochemical properties of three MAP kinases have been characterised in detail, the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK) also referred to as stress-activated protein kinase (SAPK) and the p38 MAP kinase (2). The ERK subgroup of MAP kinases is activated primarily by mitogenic stimuli such as growth factors (3). In contrast, the JNK and p38 pathways are activated primarily by a diverse array of cellular stresses including UV irradiation, hydrogen peroxide, DNA damage, heat and osmotic shock (2).

All MAP kinases are activated in response to their phosphorylation within a TXY motif, by dual specificity MAP kinase kinases (MAPKKs). The MAPKKs form a highly conserved group that are activated through phosphorylation of conserved serine and threonine residues by a somewhat more diverse group of MAP kinase kinase kinases (MAPKKKs).

In the JNK subgroup, three genes (jnk1, jnk2 and jnk3) have been described. Of these, JNK1 and JNK2 protein kinases are expressed ubiquitously, while JNK3 is expressed primarily in the brain (4). JNK is activated by two distinct MAPKKs, MKK4 (5) or MKK7 (6). A number of MAPKKKs have been identified as upstream activators of the JNK pathway, including MEKK (MEKK-1, -2, -3 and -4), the mixed-lineage protein kinases MLK-2, MLK-3, DLK and LZK (7) and Tpl-2, a member of the Raf family (8). A number of factors have been implicated in the coupling between activating stimuli and the MAPKKKs. For example, receptor-mediated signaling by growth factors involves autophosphorylation on tyrosine residues (3). Alternatively, small GTP-binding protein such as Ras (9) and members of the Rho family of GTPases, Rac and Cdc42, (10, 11, 12) have been found to couple external stimuli to MAP kinase activation. In addition, a subgroup of the Ste20-like serine/threonine protein kinases, known as germinal centre kinases (GCKs), have also been found to activate JNK through the activation of members of the MLK family (7, 13, 14).

The targeting of certain signals to specific MAP kinase modules may be regulated by the presence of scaffold proteins, such as JNK-interacting protein (JIP), which facilitates signal transduction (4). JIP has been shown to interact with the MAPKK MKK7 but not MKK4, and to interact with MAPKKKs from the mixed-lineage kinase family, but not the MAPKKK MEKK1 or MEKK4. Therefore, the function of JIP as

a scaffold protein is selective for the MLK-MKK7-JNK MAP kinase module (15). Activation of the JNK signaling pathway leads to phosphorylation of a number of targets including the transcription factors, activating transcription factor 2 (ATF-2) (16) and c-jun (17) resulting in an increase in their transcriptional activity. Overexpression of JIP proteins causes cytoplasmic retention of JNK, thereby inhibiting gene expression mediated by JNK signaling pathways (4).

Human chronic myelogenous leukemia (CML) is a malignancy of pluripotent hematopoietic cells. 95% of CML cases display a distinctive cytogenetic abnormality known as the Philadelphia (Ph) chromosome which results from a reciprocal translocation between the long arms of chromosome 9 and 22 [t(9;22) (q34;q11)] (18). This translocation results in the production of a p210 Bcr-Abl fusion protein with increased tyrosine kinase activity (19). Clinically CML follows a triphasic course; an initial chronic phase followed by an accelerated phase, which subsequently leads to blast crisis. Blast crisis is accompanied by the appearance of poorly differentiated myeloid or lymphoid blast cells in the bone marrow and is unresponsive to conventional doses of chemotherapy (20). K562 cells, which are derived from the pleural effusion of a patient in terminal blast crisis and express the p210 Bcr-Abl fusion protein (21), are particularly resistant to the induction of apoptosis by various agents including camptothecin, ara-C, etoposide, paclitaxel, staurosporine and anti-Fas antibodies (22). Downregulation of Bcr-Abl using anti-sense treatment restores the sensitivity of CML cells to apoptosis-inducing chemotherapeutic agents (23). We have recently found that some members of a novel series of pyrrolo-1,5-benzoxazepines potently induce apoptosis in a number of CML cell lines, such as K562, KYO.1 and LAMA 84, by bypassing the apoptotic suppressor, Bcr-Abl (24). An insight into the mechanism of action of many novel drugs has helped in establishing their therapeutic potential in the treatment of various diseases. In this study we sought to investigate the mechanism by which a representative compound from this series, known as PBOX-6, induces apoptosis in CML cells. We provide evidence that activation of the stress activated protein kinase, JNK, is an essential part of the mechanism by which this compound induces apoptosis. Apoptosis is accompanied by phosphorylation of the JNK substrates, c-Jun and ATF-2. We show that PBOX-6-induced apoptosis occurs independently of small G-proteins, Ras, Rac and Cdc42 and is likely to involve phosphorylation and activation of MKK-7 and members of the MLK and GCK families, which occurs upstream of JNK activation.

EXPERIMENTAL PROCEDURES

Materials

K562 human chronic myelogenous leukemia cells were gratefully received from Dr. Mark Lawler (St. James's Hospital, Dublin). The pyrrolobenzoxazepine 7-[(dimethylcarbamoyl)oxy]-6-(2-naphthyl)pyrrolo-[2,1-*d*][1,5]-benzoxazepine (PBOX-6) was synthesised as described previously (25). The RapiDiff kit was obtained from Diagnostic Developments (Burscough, Lancashire, UK). FuGENE 6 transfection reagents was from Roche Molecular Biochemicals (Mannheim, Germany). Anti-JNK, anti-ERK and anti-p38-phospho-specific polyclonal antibodies, anti-ATF-2-phospho-specific and anti-c-jun-phospho specific antibodies were purchased from New England Biolabs (Hertfordshire, United Kingdom). Dicoumarol was obtained from Sigma (Poole, Dorset, UK). The ATF-2- and c-Jun-luciferase reporting systems, (PathDetect^{TM}) were purchased from Stratagene (La Jolla, California). The constitutively active mutants (RacV12 and RasV12), and dominant negative (DN) mutants (RacN17 and RasN17), along with the JIP-1 plasmid and the β-galactosidase expression vector were kindly provided by Professor Luke O'Neill (Biochemistry Department, Trinity College Dublin). Wild type and dominant negative versions of cdc42 were generous gifts from Professor Alan Hall (University College, London, United Kingdom). Unless stipulated, all other reagents were from Sigma.

Cell culture and induction of apoptosis

K562 cells were cultured in RPMI-1640 medium supplemented with 10% foetal calf serum, gentamycin (0.1mg/ml), and L-glutamate (2mM) and incubated in a humidified atmosphere of 95% O_2 and 5% CO_2 at 37^oC. Cells were seeded at a density of 3 x 10^5 cells/ml and following treatment with PBOX-6 (10μM), an aliquot (100μl) was cytocentrifuged onto glass slides. They were then stained with the RapiDiff kit (eosin/methylene blue) under conditions described by the manufacturer. The degree of apoptosis and necrosis was determined by counting approximately 300 cells under a light microscope. At least 3 fields of view per slide, with an average of approximately 100 cells per field, were counted and the percent apoptosis and necrosis was determined as previously described (26).

Transfections

Transient transfection of K562 cells was performed with FuGENE 6 transfection reagent, using 400ng DNA in 100μl of serum free RPMI-1640 medium combined with 1.5μl FuGENE 6 in 20μl serum free

RPMI-1640. The DNA/FuGENE mixture was incubated for 15 min at room temperature and was then added dropwise to 4 x 10^5 cells in 400µl RPMI-1640 containing 20% foetal calf serum, in a 24 well dish. Following overnight incubation, cells were stimulated with either vehicle (1% (v/v) ethanol) or PBOX-6 (10µM). Cells were harvested for luciferase and β-galactosidase assays. A luciferase assay were performed by centrifugation of the cells at 500xg for 5 min, and the pellets were lysed using passive lysis buffer (Promega) followed by gentle agitation at room temperature for 15 min. Luciferase activity was assayed by the addition of luciferase assay mix (40µl) (20mM tricine, 1.07mM $(MgCO_3)_4Mg(OH)_2.5H_20$, 2.67 mM $MgSO_4$, 0.1mM EDTA, 33.3mM DTT, 270mM coenzyme A, 470mM luciferin, 530mM ATP) to each sample and luminescence was read using a luminometer. β-galactosidase activity was assayed by the addition of o-nitrophenylgalactosidase to an aliquot of cell lysate (20µl) in a 96 well plate followed by the addition of β-galactosidase buffer (23mM NaH_2PO_4, 77mM Na_2HPO_4, 0.1mM $MnCl_2$, 2mM $MgSO_4$, 40mM β-mercaptoethanol pH 7.3) and the plate was incubated at 37°C overnight and absorbance was read at 405nm. All transfections were performed at least in triplicate, and all luciferase values were normalized according to β-galactosidase readings.

Immunoblotting

Cells were collected by centrifugation at 500xg, washed with ice-cold phosphate buffered saline and lysed on ice for 20 min in a buffer containing 150mM NaCl, 50mM Tris/Cl pH 8.0, 0.1% (v/v) SDS, 1.0% (v/v) Triton X-100, sodium orthovanadate (1mM), phenylmethylsulfonyl fluoride (1mM) supplemented with leupeptin (1µg/ml), and aprotinin (10µg/ml), followed by centrifugation at 20,000xg for 10 min and the supernatants were collected. Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidiene difluoride (PVDF) membrane by electroblotting. Membranes were probed with phospho-specific antibodies to JNK, ERK, p38, ATF-2 or c-jun according to the manufacturers' instructions. Proteins were visualised using enhanced chemiluminesence reageants (ECL from Amersham).

RESULTS

PBOX-6 induces the transient activation of two JNK isoforms, JNK 1 and JNK 2, in K562 cells-Mitogen-activated protein (MAP) kinases represent one of the most important signaling cascades in response to extracellular stimuli and it is the dual phosphorylation of MAP kinases by upstream kinases that results in their activation (1). The ability of PBOX-6 to activate intracellular MAP kinase pathways in K562 cells was assessed following treatment with either vehicle or PBOX-6 for various lengths of time. Whole cell extracts were prepared and Western blotting was performed using antibodies against the active/phosphorylated form of the MAP kinases, ERK, JNK and p38. Fig. 1A demonstrates that PBOX-6 induces the transient activation of two JNK isoforms, JNK1 and JNK2, in K562 cells. JNK activation becomes visible following a 15 min treatment with PBOX-6, peaks between 30-45 min and slowly declines to basal levels over 8h. In contrast, whereas UV irradiation of Jurkat cells for 2 min activates p38, PBOX-6 treatment of K562 cells for up to 8h fails to activate p38 (Fig.1B). Similarly, PMA treatment of K562 cells for 30 min activates the ERK MAP kinase whereas PBOX-6 treatment of cells for up to 8h has no effect (Fig. 1C). These results suggest that activation of the JNK MAP kinase, and not the p38 or ERK MAP kinase, may be an important intermediate in the pathway by which PBOX-6 induces apoptosis in K562 cells. Furthermore, it has been previously shown that only some members of this series of pyrrolo-1,5-benzoxazepines induce apoptosis in CML cells (24). In this study we have found that the pro-apoptotic members result in activation of JNK whereas the non-apoptotic members failed to activate JNK in K562 cells (data not shown).

The JNK substrates, c-jun and ATF-2, become activated in K562 cells in response to PBOX-6- It is widely reported that the JNK MAP kinase phosphorylates the transcription factors c-jun and ATF-2 resulting in increased transcriptional activity (3, 16, 27). Results from Western blotting using phospho-specific antibodies demonstrate that PBOX-6 induces a dose- and time-dependent phosphorylation of c-jun (Fig. 2 A and B) and ATF-2 (Fig. 2 C and D) in K562 cells. Taken together these results demonstrate that PBOX-6-induced activation of JNK and its downstream substrates, c-jun and ATF-2, in K562 cells, may be important in the mechanism in which this compound induces apoptosis.

The JNK inhibitior, dicoumarol, prevents activation of c-jun and ATF-2 and abolishes PBOX-6-induced apoptosis in K562 cells- It has recently been shown that the quinone reductase inhibitor, dicoumarol,

specifically inhibits activation of the JNK MAP kinase in response to a variety of stress stimuli (28, 29). In order to determine whether activation of JNK is directly associated with the pro-apoptotic activity of PBOX-6 in K562 cells, we sought to block JNK activity using dicoumarol and to determine the effect on the extent of apoptosis induced by PBOX-6. Cells were treated with PBOX-6 (10μM) for 16h in the absence and presence of dicoumarol and cell lysates were prepared. Western blot analysis reveals that dicoumarol inhibits c-jun and ATF-2 phosphorylation in response to PBOX-6 (Fig. 3 A). In order to determine the effect of dicoumarol on PBOX-6-induced apoptosis, K562 cells were pretreated with dicoumarol for 1h prior to treatment with PBOX-6 for a further 16h, followed by RapiDiff staining of the cells and the extent of apoptosis was determined by morphological examination. Results shown in Fig. 3 B illustrate that although dicoumarol itself does not induce apoptosis in K562 cells, however, pretreatment with dicoumarol completely abolishes PBOX-6-induced apoptosis in these cells. These results confirm earlier findings that dicoumarol inhibits JNK activity and suggests that c-jun and ATF-2 phosphorylation in K562 cells occurs as a direct consequence of JNK activation. In addition, the ability of dicoumarol to completely abrogate apoptosis suggests that JNK activation is essential in the pathway by which PBOX-6 induces apoptosis in K562 cells.

JIP-1 inhibits c-jun and ATF-2 phosphorylation, and apoptosis induced by PBOX-6 in K562 cells-In order to confirm the importance of JNK activation during PBOX-6-induced apoptosis and to further delineate the signal transduction cascade induced, transient transfection assays using a luciferase reporting system were carried out. Overexpression of the JNK scaffold protein, JIP-1, has been previously shown to inhibit the downstream signaling of JNK (4).

K562 cells were transiently transfected with a c-jun or ATF-2 dependent luciferase reporting system. Activation of c-jun and ATF-2, expressed as fold stimulation over unstimulated cells, shows an approximate 4-fold increase in c-jun activity and a 7-fold increase in ATF-2 activity in response to PBOX-6 treatment, whereas ethanol (1%) had no effect (Fig. 4 A). These results confirm the earlier findings from Western blotting outlining that PBOX-6 causes phosphorylation of the JNK substrates, c-jun and ATF-2. To confirm the essential requirement of JNK activation during PBOX-6-induced apoptosis, K562 cells, transfected with the ATF-2 luciferase reporting system, were co-transfected with JIP-1 and the effect on PBOX-6 activity was determined. Results shown in Fig. 4 B demonstrate that while PBOX-6 stimulates a 5-fold increase in ATF-2-dependent reporter gene activity, co-transfection of the JIP-1 protein into K562

cells completely inhibits PBOX-6-induced stimulation of ATF-2 activity. In addition, K562 cells were transfected with empty vector ($PCDNA_3$) or JIP-1 followed by treatment with PBOX-6 for a further 16h and the extent of apoptosis was determined by morphological examination. Results shown in Fig. 4 C demonstrate that co-transfection of JIP-1 into K562 cells significantly reduces the extent of apoptosis induced by PBOX-6, which is consistent with a 50% transfection efficiency. These findings confirm earlier results, which suggest that JNK activation is essential during PBOX-6-induced apoptosis in K562 cells.

*PBOX-6-induced activation of JNK in K562 cells occurs independently of Ras, Rac and Cdc42-*A number of upstream components are implicated in activation of the JNK signaling cascade, including the small GTP-binding proteins Ras, Rac and Cdc42 (9, 10, 12, 30). In order to determine whether these small G-proteins are activated upstream of JNK during PBOX-6-induced apoptosis in K562 cells, cells were co-transfected with constitutively active (V12) and dominant negative (N17) mutants of these proteins and the effect on PBOX-6-induced phosphorylation of ATF-2-dependent luciferase reporter gene was determined. Results shown in Fig. 5 indicate that co-transfection with constitutively active versions of Ras, Rac and Cdc42 cause an increase in ATF-2 phosphorylation, confirming that these proteins are capable of activating the JNK signaling pathway. Upon treatment of cells with PBOX-6 an additive increase in ATF-2 activity is observed as PBOX-6 also drives the JNK signaling pathway. On the other hand, cells transfected with RasN17, RacN17 or Cdc42N17 alone have no effect on ATF-2 activation, whereas following treatment with PBOX-6, DN mutants fail to inhibit PBOX-6-induced activation of ATF-2, suggesting that activation of these small G-proteins does not lie upstream of JNK in the signal transduction pathway by which PBOX-6 induces apoptosis in K562 cells.

DISCUSSION

The results presented herein provide an insight into the mechanism whereby a novel apoptotic agent, known as PBOX-6, induces apoptosis in the extremely drug-resistant K562 cell line. PBOX-6 was found to activate the JNK signaling pathway, but not the ERK or p38 MAP kinases pathways, in CML cells. Although the three MAP kinase pathways share structural similarities, the outcome of activation is quite different. ERK stimulation by mitogenic and trophic agents results in cell division and differentiation whereas activation of p38 and JNK results in growth arrest and cell death (31). The same signaling pathway can often have different functions depending on the cell context. However, since JNK is activated by a variety of cellular stresses, it has been proposed to serve as the major apoptosis switch. For example, activation of the JNK signaling pathway has been shown to result in apoptosis in response to cis-platinum and heat shock (31), DNA damaging agents such as anisomycin and UV irradiation (28, 32), chemotherapeutic agents such as etoposide and camptothecin (17) and vinblastine and adriamycin (33).

Pathologically, failure of an apoptosis programme often leads to an imbalance in cell number which in turn leads to tumourigenesis. Therefore, control of apoptosis has emerged as an important strategy for clinical cancer therapy (17). Although a number of chemotherapeutic agents have been used in the treatment of leukemia, many forms such as chronic myeloid leukemia are resistant to the induction of apoptosis (22). Recently we have shown that some members of a novel series of pyrrolo-1,5-benzoxazepine compounds potently induce apoptosis in CML cells (24), and they do so by bypassing the apoptotic suppressor Bcr-Abl, indicating the potential of these novel compounds in the treatment of CML and related disorders. In the present study we set out to delineate the apoptotic signaling pathway induced in CML cells by a representative compound from this novel series, called PBOX-6. Our findings that PBOX-6 induces that transient activation of two JNK isoforms, JNK1 and JNK2, in K562 cells supports a role for this stress pathway in the induction of apoptosis by PBOX-6. The quinone reductase inhibitor, dicoumarol, has been shown to inhibit JNK activation in response to the TNFα receptor interacting protein, TRAF2, and in response to anisomycin, sorbitol, UV irradiation and ceramide, while having no effect on the mitogen-activated pathways (28). In this study, inhibition of JNK activity in K562 cells by dicoumarol, together with its inhibitory effect on the extent of apoptosis induced by PBOX-6 confirms the importance of JNK activation in the apoptotic pathway induced by PBOX-6. In addition, we have previously reported that only some members of this novel series of pyrrolo-1,5-benzoxazepines induce apoptosis in CML cells (24). We have found that all the members that induce apoptosis in CML cells result in activation of JNK, whereas the

non-apoptotic members failed to activate JNK (data not shown), lending further support to the suggestion that JNK activation, which occurs within 15 min, is an important part of the mechanism by which these novel compounds induce apoptosis in CML cells. The phosphorylation of several transcription factors, such as c-jun and ATF-2, in response to JNK activation, has been well documented. In agreement with this, PBOX-6 induces phosphorylation and thus activation of c-jun and ATF-2, in K562 cells, in a time-and dose-dependent manner, which further outlines the importance of the JNK signaling pathway during PBOX-6-induced apoptosis in K562 cells.

JNK activation occurs due to dual phosphorylation by the upstream kinases, MKK4 or MKK7. These are phosphorylated and activated by further upstream kinases known as MAPKKK. A number of different MAPKKK have been shown to activate JNK and these include members of the MEKK and MLK families. For example it has been shown that overexpression of MLK-3 was sufficient to activate JNK in COS-7 cells (12). Although it is clear that the MLKs play an important role in transmitting a variety of different signals to JNK activation, the mechanisms that directly regulate MLK activity are not well understood. It is however believed that in some cases, members of the MLK family are regulated by the small GTP-binding proteins, Rac and Cdc42, and by a subgroup of the ste20-related kinase family known as germinal centre kinases (GCKs) (34). For example, MLK has been shown to bind Cdc42 and Rac *in vivo*, through a Cdc42/Rac interactive binding domain (CRIB), and a dominant negative mutant of MLK3 abolishes activation of JNK by Cdc42 and Rac (12). In addition, a GCK family member known as HGK has been found to induce JNK activation in human embryonic kidney cells, which was inhibited by dominant negative MKK4 and MKK7 mutants (6). In the present study we found that the small GTP-binding proteins, Ras, Rac and Cdc42 are not involved in the upstream signaling pathway leading to JNK activation upon PBOX-6 treatment of K562 cells. In addition, the synergy observed between constitutively active G-proteins and PBOX-6 would suggest that the GTPases and PBOX-6 activate JNK via independent signaling pathways. These results are in agreement with many reports, which demonstrate that JNK activation may occur independently of the GTP-binding proteins (9, 35, 36). For example, dominant negative Rac and Cdc42 had no effect on anisomycin-induced JNK activation in COS-7 cells (35), or on IL-1 and PDGF-induced JNK activation in PAE cells (36). Therefore, it seems that GTPases may only modulate JNK activity in certain cell types and under certain conditions. In support of this concept, the MLK family members DLK and LZK have been shown to lack a CRIB domain suggesting that MLKs can be regulated in a GTPase independent pathway (37). Germinal centre kinases which are located upstream

of JNK, lack a CRIB domain and are therefore not under the control of the small GTPases. It has indeed been reported that dominant negative forms of MLK-3 block JNK activation mediated through the Ste20 homologues GCK and HPK (7).

JIP-1 has been identified as a scaffold protein that interacts in a specific fashion with specific kinases and leads to activation of the JNK signaling pathway. Components of the complex mediated by JIP-1 have been used to determine the specificity of the stress activated kinase signaling pathway. In addition, overexpression of JIP-1 inhibits the downstream JNK signaling pathway, due to cytoplasmic retention of the JNK signaling module (4). Data obtained in the present study using JIP-1 has been used to delineate the signaling pathway induced by PBOX-6. We have found that co-transfection of JIP-1 into K562 cells completely abolishes PBOX-6-induced JNK activity and inhibits apoptosis. This data correlates with results obtained earlier using dicoumarol which indicate that JNK activity is essential during PBOX-6-induced apoptosis in K562 cells. JIP-1 selectively binds components of the JNK signaling module. It has been shown that JIP-1 binds to members of the MLK family, but not to MEKK1 or MEKK4 (15). In addition, JIP-1 interacts with HPK1, a member of the GCK family, MKK7 from the MAPKK tier of kinases but it does not interact with MKK4 or the small GTP-binding proteins, Rac and Cdc42 (4, 15). Consistent with this, it has been reported that although MLK-3 and MLK-2 can activate JNK via MKK4 and MKK7, these kinases show preferential association with MKK7. In addition, DLK activates JNK through MKK7 only (7). Therefore JIP-1 selectively scaffolds the MLK-MKK7-JNK kinase module. The ability of JIP-1 to block PBOX-6-induced apoptosis in K562 cells supports the finding that apoptosis occurs in a GTPase-independent fashion and strongly supports the hypothesis that members of the GCK and MLK families, together with MKK7 are involved in the upstream apoptotic pathway induced by PBOX-6 in K562 cells. Events occurring upstream of GCKs and leading to JNK activation have not been fully determined, however, some reports suggest that activation of GCK family members occurs following recruitment, via adaptor proteins, to receptor tyrosine kinases such as the epidermal growth factor receptor (38). Further work to investigate this hypothesis is currently underway.

In summary, we have found that the novel apoptotic agent, PBOX-6, induces the transient activation of the JNK signaling pathway in K562 cells, an event that is crucial for its apoptotic activity. In an attempt to further delineate the signaling pathway activated by PBOX-6, we conclude that apoptosis occurs independently of the GTP-binding proteins Ras, Rac and Cdc42 and is likely to involve activation of members of the GCK and MLK families, together with MKK7, thus leading to JNK activation.

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Footnotes:

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Abbreviations:

PBOX, pyrrolo-1,5-benzoxazepine; CML, chronic myelogenous leukemia; MAP, mitogen-activated protein; JNK, c-jun NH_2-terminal kinase; ATF-2, activating transcription factor 2; JIP, JNK-interacting protein; MLK, mixed-lineage kinase; GCK, germinal centre kinase

Figure legends

Fig. 1. Effect of PBOX-6 on JNK, p38 and ERK MAP kinase activation. K562 cells were seeded at $5x10^6$cells and treated with vehicle (1% (v/v) ethanol) or PBOX-6 (10µM) for up to 8h. Whole cell extracts were prepared and equal amounts of protein (30µg) were resolved on a 10% SDS-PAGE gel and transferred onto PVDF. Membranes were probed overnight with *A*, phospho-specific JNK antibody *B,* phospho-specific p38 antibody or *C,* phospho-specific ERK antibody. In *B,* a positive control in lane 1 consists of Jurkat cells, which were UV irradiated for 2 min and incubated at 37°C for a further 2h, whereas in *C,* a positive control in lane 1 consists of K562 cells that were treated with PMA (100nM) for 30 min. Results are representative of two separate experiments.

Fig. 2. Dose- and time-dependent phosphorylation of c-jun and ATF-2 by PBOX-6. K562 cells ($5x10^6$) were treated with vehicle (1% (v/v) ethanol) or (*A and C*) a range (0.1, 1, 5 and 10µM) of PBOX-6 concentrations for 16h or (*B and D*) PBOX-6 (10µM) for 1, 4, 8 and 16h. Whole cell extracts were prepared and protein (30µg) was resolved on a 10% SDS-PAGE gel followed by Western blotting. Membranes were probed with either (*A and B*) phospho-specific c-jun antibody or (*C and D*) phospho-specific ATF-2 antibody. Blots were stripped by incubating for 30 min in 62.5mM Tris, pH 7.8, 100mM β-mercaptoethanol and 2% (v/v) SDS at 50°C, and re-probed with an antibody against β-actin as a loading control. Results are representative of two separate experiments.

Fig. 3. Inhibition of PBOX-6-induced c-jun and ATF-2 phosphorylation, and apoptosis, by dicoumarol. K562 cells were treated with either vehicle (1% (v/v) ethanol) for 17h, dicoumarol (200µM) for 17h, PBOX-6 (10µM) for 16h, or a pretreatment of dicoumarol (200µM) for 1h prior to treatment with PBOX-6 (10µM) for a further 16h. In *A*, whole cell lysates were prepared and an equal amount of protein (30µg) was resolved on a 10% SDS-PAGE gel followed by Western blotting. Membranes with incubated with phospho-specific c-jun antibody (*upper panel*), subsequently stripped and re-probed with phospho-specific ATF-2 antibody (*middle panel*). Blots were stripped again and re-probed with β-actin as a loading control (*lower panel*). In *B*, the extent of apoptosis was determined by centrifuging an aliquot of cells (100µl) onto a slide and staining with the RapiDiff kit as outlined in Experimental Procedures. Results are representative of three separate experiments.

Fig. 4. Effect of JIP-1 on JNK activity and apoptosis induced by PBOX-6. *A,* K562 cells were transiently transfected, using the FuGENE 6 reagent, with the c-jun- or ATF-2-dependent luciferase reporter genes and the β-galactosidase expression vector (400ng in total), followed by treatment with vehicle (1% (v/v) ethanol) or PBOX-6 (10μM) for 48h. Cells were lysed and reporter gene activity was determined as a function of luciferase activity, and was normalized according to β-galactosidase readings. In *B,C* K562 cells were transiently transfected with the ATF-2-luciferase reporter gene and the β-galactosidase vector, together with co-transfection with either empty vector ($PCDNA_3$) or JIP-1. In *B*, cells were treated with vehicle (1% (v/v) ethanol) or PBOX-6 (10μM) for 48h and ATF-2 activity was expressed as a function of luciferase activity. All results are expressed as fold stimulation over unstimulated cells and represent the mean +/- S.E.M. of triplicate determinations performed three times. In *C*, cells were treated with vehicle (1% (v/v) ethanol) or PBOX-6 (10μM) for 16h and the extent of apoptosis was determined by RapiDiff staining followed by morphological examination as outlined in Experimental Procedures. Results represent the mean +/- S.E.M. of three separate experiments. Statistical analysis was carried out using the Instat computer program. * $p<0.001$ with respect to vehicle treatment; Students t-test.

Fig. 5. Effect of constitutively active and dominant negative mutants of Ras, Rac and Cdc42 on PBOX-6 induced activation of ATF-2-dependent luciferase reporter gene. K562 cells were transiently transfected with the ATF-2-luciferase reporting system and a β-galactosidase expression vector, and in some cases, an empty vector ($PCDNA_3$) or a plasmid encoding either *A*, RasV12 or RasN17, *B,* RacV12 or RacN17, *C*, Cdc42V12 or Cdc42N17. Cells were treated with either vehicle (1% (v/v) ethanol) or PBOX-6 (10μM) for 48h. ATF-2 activity was determined as a function of luciferase activity. Results are expressed as fold stimulation over vehicle treated cells and represent the mean +/- S.E.M. of triplicate determinations performed three times.

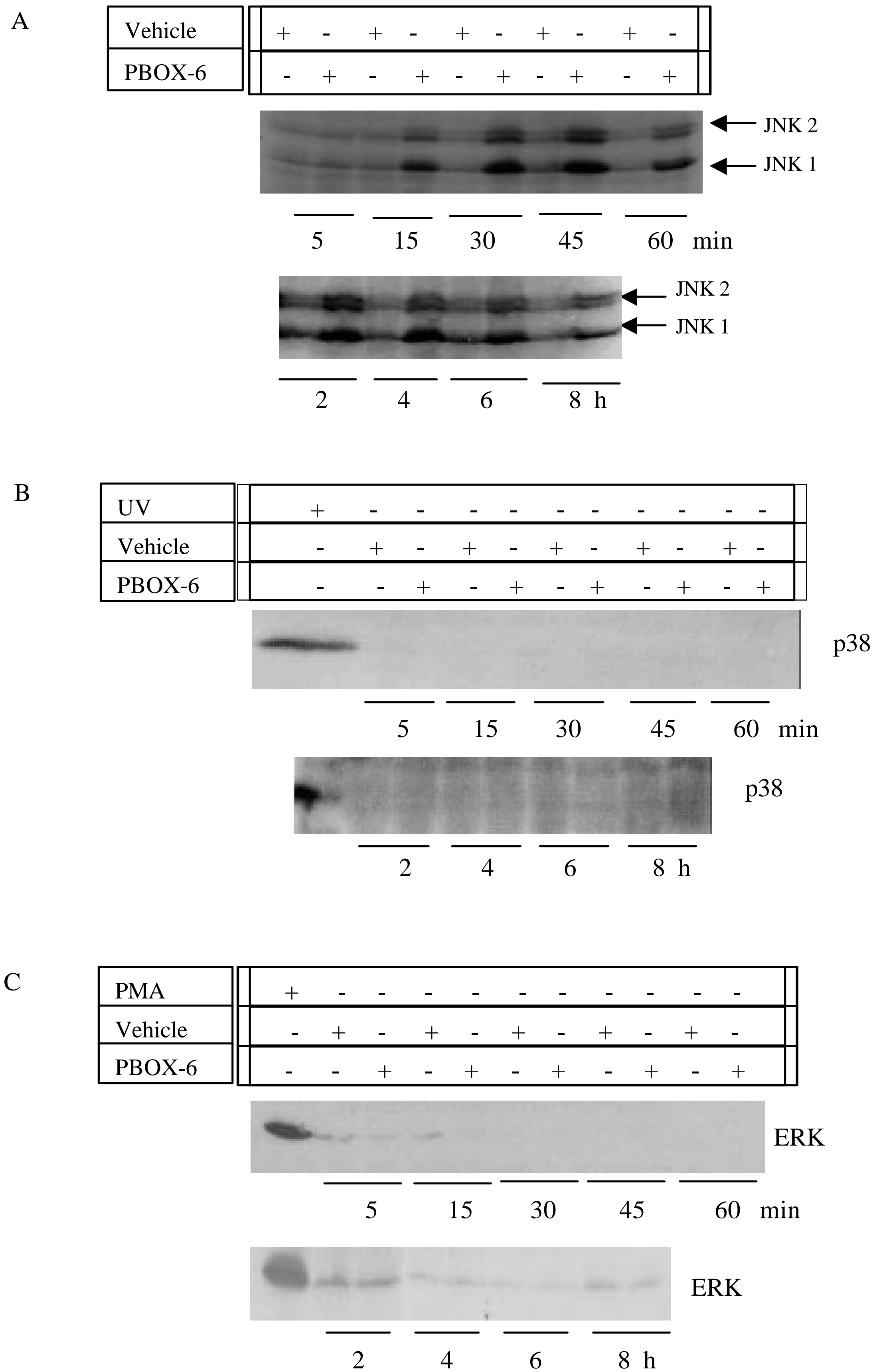


Fig. 1. Mc Gee et al.

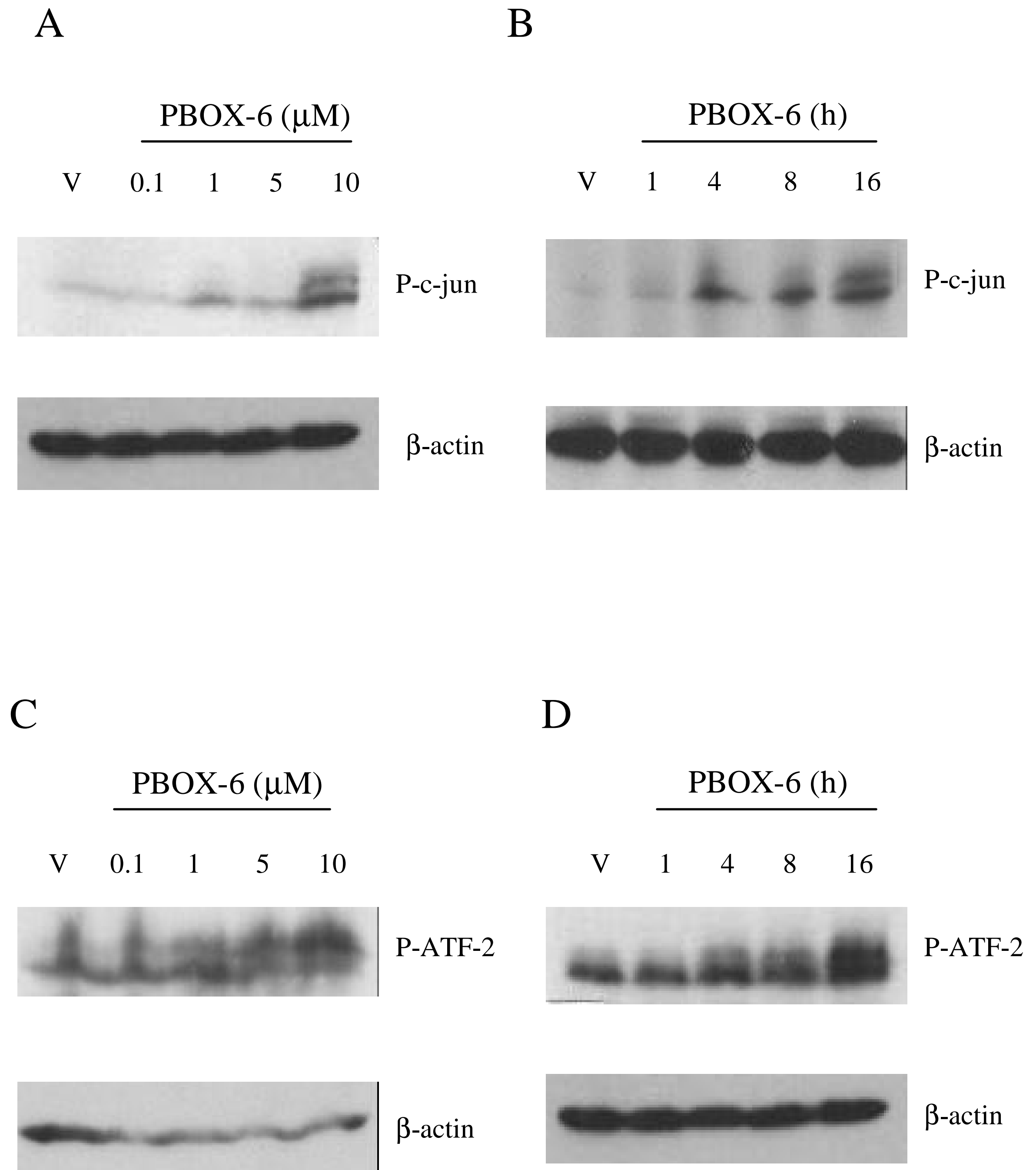
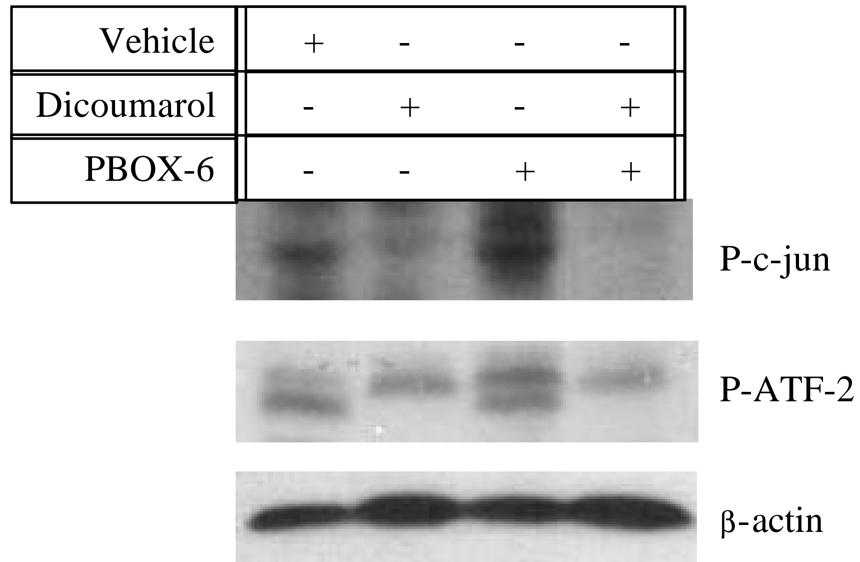


Fig. 2. Mc Gee et al.

A



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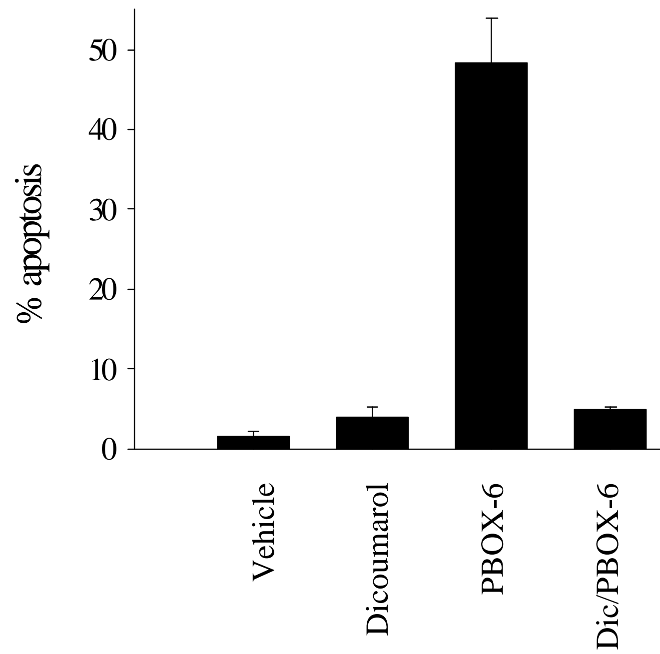

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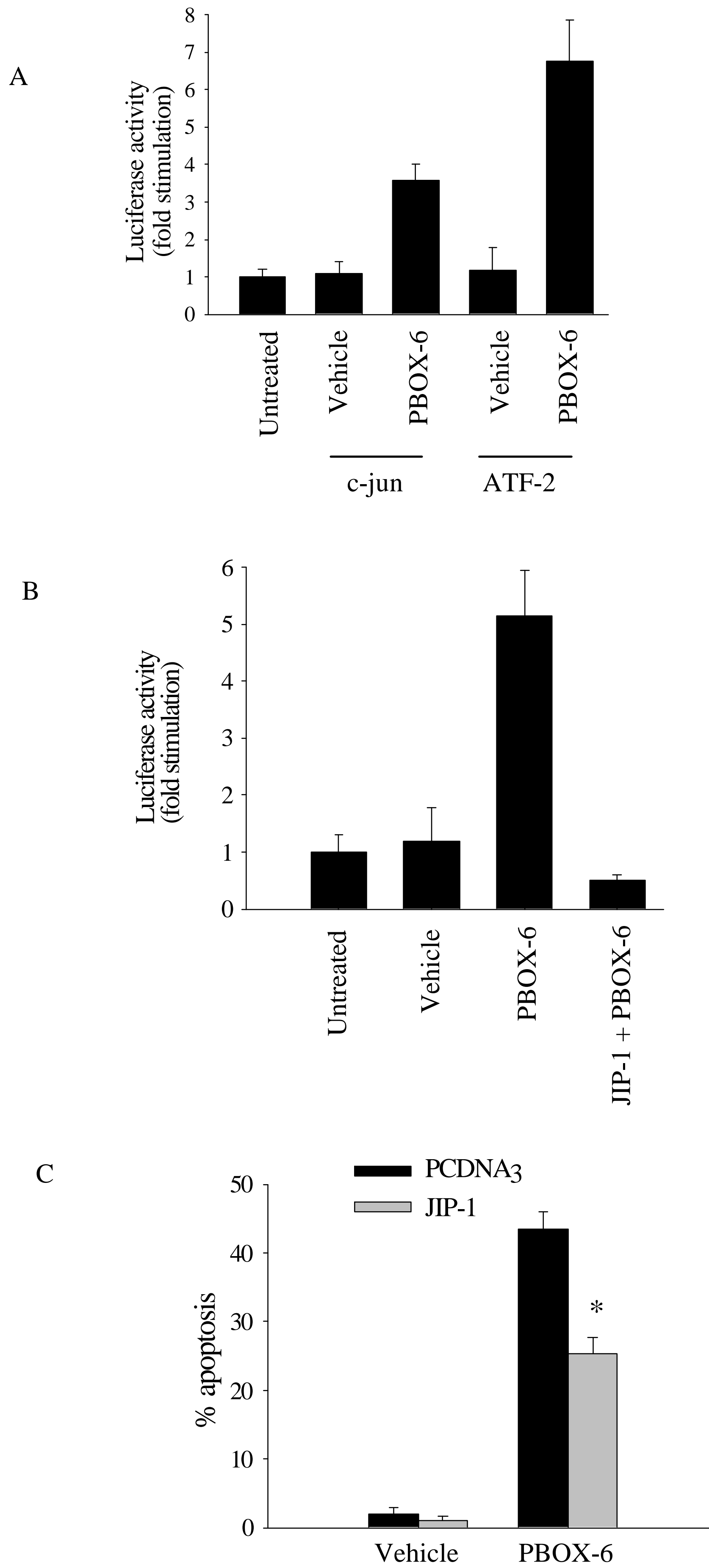

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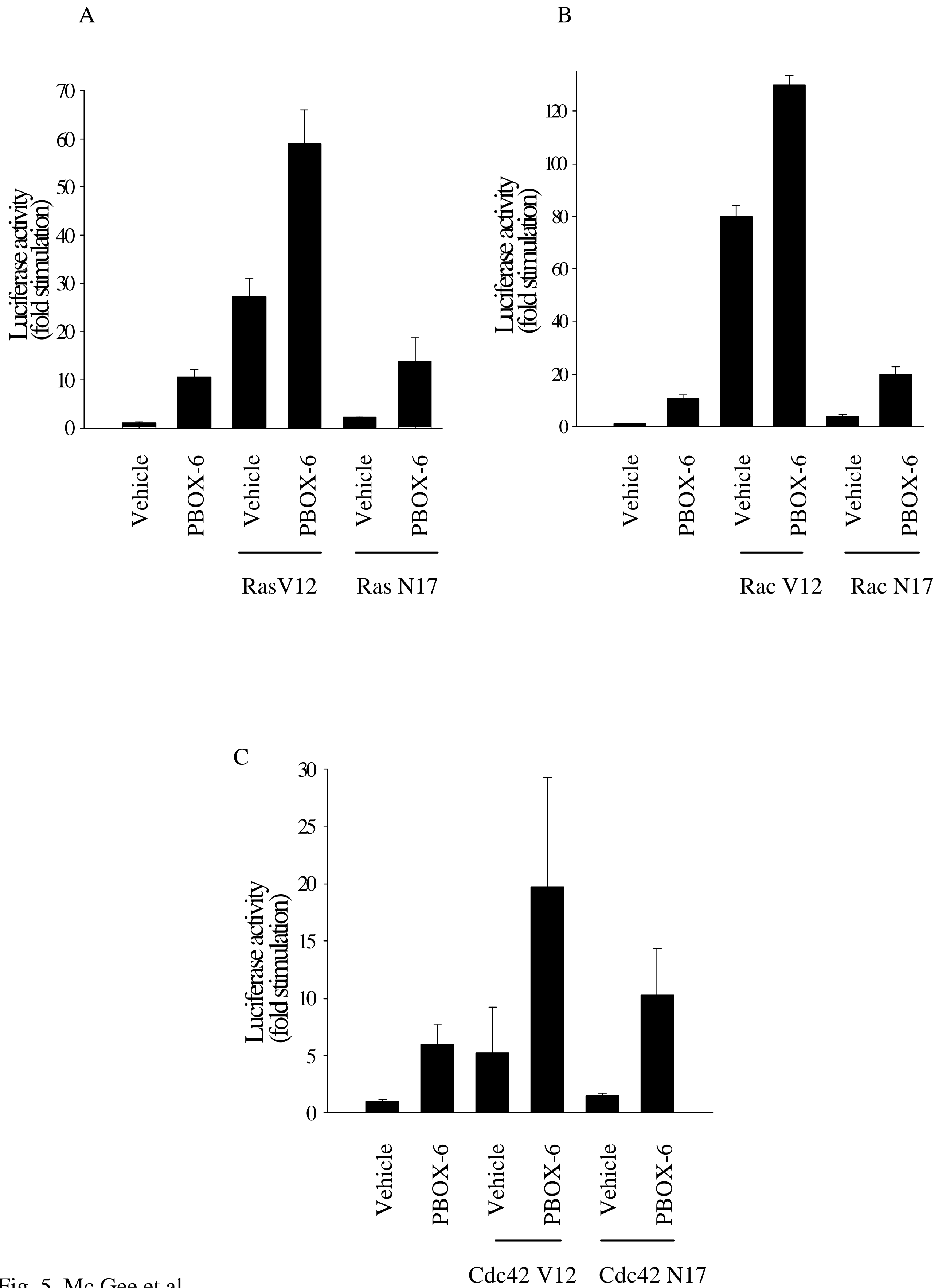

Fig. 5. Mc Gee et al.