The Selective Protein Kinase C Inhibitor, Ro-31-8220, Inhibits Mitogen-activated Protein Kinase Phosphatase-1 (MKP-1) Expression, Induces c-Jun Expression, and Activates Jun N-terminal Kinase*

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Jerlyn Beltman‡§, Frank McCormick, and Simon J. Cook§

From ONYX Pharmaceuticals, 3031 Research Drive, Richmond, California 94806

The role of protein kinase C (PKC) in inflammation, mitogenesis, and differentiation has been deduced in part through the use of a variety of PKC inhibitors. Two widely used inhibitors are the structurally related compounds GF109203X and Ro-31-8220, both of which potently inhibit PKC activity and are believed to be highly selective. While using GF109203X and Ro-31-8220 to address the role of PKC in immediate early gene expression, we observed striking differential effects by each of these two compounds. Growth factors induce the expression of the immediate early gene products MAP kinase phosphatase-1 (MKP-1), c-Fos and c-Jun. Ro-31-8220 inhibits growth factor-stimulated expression of MKP-1 and c-Fos but strongly stimulated c-Jun expression, even in the absence of growth factors. GF109203X displays none of these properties.

These data suggest that Ro-31-8220 may have other pharmacological actions in addition to PKC inhibition. Indeed, Ro-31-8220 strongly stimulates the stress-activated protein kinase, JNK1. Furthermore, Ro-31-8220 apparently activates JNK in a PKC-independent manner. Neither the down-regulation of PKC by phorbol esters nor the inhibition of PKC by GF109203X affected the ability of Ro-31-8220 to activate JNK1. These data suggest that, in addition to potently inhibiting PKC, Ro-31-8220 exhibits novel pharmacological properties which are independent of its ability to inhibit PKC.

Protein kinase C (PKC) is a large and diverse family of protein kinases, whose members are differentially regulated by calcium, phorbol esters, diglycerides, and phosphatidic acid (for a review, see Ref. 1). The various PKC isozymes play important signaling roles in cellular growth, differentiation, and homeostasis. The reported cellular roles of PKC have been identified largely through the use of PKC activators and inhibitors.

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§ To whom correspondence should be addressed: ONYX Pharmaceuticals, 3031 Research Dr., Richmond, CA 94806. Tel.: 510-222-9700; Fax: 510-222-9758; E-mail: jerlyn@onyx-pharm.com or simon@onyx-pharm.com.
1 The abbreviations used are: PKC, protein kinase C; ERK, extracellular signal regulated kinase; JNK, Jun N-terminal kinase; MKP-1, MAP kinase phosphatase-1; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; MEK, MAPK or ERK kinase; ET-1, endothelin-1; PAGE, polyacrylamide gel electrophoresis; MBP, myelin basic protein; SEK, SAPK or ERK kinase; GST, glutathione S-transferase.

2 K. A. Cadwallader, J. Beltman, F. McCormick, and S. J. Cook, submitted for publication.
signaling cascades, ERK signaling can be antagonized through cross-talk by a cAMP-dependent pathway (14–16) and is inactivated by the phosphatase MKP-1, an immediate early gene whose expression is induced by growth factors (17). Most transriptional events are tightly regulated by the convergence of multiple signals. Therefore, the regulation of MKP-1 expression is probably complex and tightly regulated by the contribution of multiple pathways. The observation that the PKC agonist, PMA, can activate ERK but that no sustained phase is observed suggests that PKC, in at least some instances, may contribute to the regulation of MKP-1 expression (43). Indeed, MKP-1 is induced by PMA (18, 19).

PKC is known to regulate the expression of a variety of immediate early genes, such as c-Fos and c-Jun. In the course of studying the role of PKC in regulating MKP-1 expression, we noted striking differences in the properties of two widely used PKC inhibitors, Ro-31-8220 and GF109203X. This led us to speculate that Ro-31-8220 possessed additional unique properties which might be dissociable from PKC inhibition. Here we show that Ro-31-8220 is able to inhibit MKP-1 expression, stimulate c-Jun expression, and activate JNK1. These properties are observed at doses and time points frequently used to inhibit PKC in whole cells.

**EXPERIMENTAL PROCEDURES**

**Materials—** All cell culture reagents were from Irvine Scientific. [γ-32P]ATP and [3H]leucine were from DuPont NEN. LPA was from Avanti Polar lipids. EGFr and protease inhibitors were from Boehringer Mannheim. Ro-31-8220 and GF109203X were from LC Laboratories. Precast SDS-PAGE gels were from Novex Gel System. The E1.2 peptide antisemur to ERK1 was that of Cook et al. (20). Peptide antiserum against JNK1 was from Santa Cruz Biotechnology. The GST-c-Jun fusion protein expression plasmid was obtained from Hibi et al. (7). All other reagents including MBP, Protein A-Sepharose, and PMA were from Sigma.

**Cell Culture, Stimulation, and Lysates—** Rat-1 fibroblasts were maintained at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 0.6 mg/ml glucose, 100 units/ml penicillin and streptomycin, and 10% fetal bovine serum. Confluent 6-cm cultures of Rat-1 fibroblasts were washed once in serum-free media and then maintained in serum-free media for 18 h prior to the application of external stimuli. Cells were then stimulated and PKC was down-regulated or inhibited by the growth factors and other agents directly to the serum-free media. Time courses were carried out at 37 °C, and the reaction was stopped by aspirating the media and replacing it with 700 μl of ice-cold lysis buffer (20 mM Tris, pH 7.6, 0.5% Triton X-100, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 10 μg/ml Pefabloc SC, 2 mM sodium vanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM diithiothreitol). Cell lysates were then prepared by rocking the cell culture dishes at 4 °C for 20 min and collecting the resulting lysate which was then clarified by spinning at 10,000 rpm for 10 min.

**Immune-complex Kinase Assays—** 300 μl of the aforementioned lysate is sufficient for a single immunoprecipitation. Equal quantities of lysate were used for ERK and JNK immunoprecipitation with 40 μl of Protein A-Sepharose (30% slurry) and 3 μl of crude E1.2 crude serum or 4 μl of anti-JNK1 serum. Immunoprecipitations took place with end-over-end rotation at 4 °C for 2 h. The resulting immune-complex was then recovered by centrifugation, washed with lysis buffer, and then with ERK or JNK kinase buffer (for ERK: 30 mM Tris, pH 8.0, 20 mM MgCl2, 2 mM MnCl2, 1 mM DTT, 2 μM ATP, 10 μg/ml phosphatase substrate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM diithiothreitol). Cell lysates were then prepared by rocking the cell culture dishes at 4 °C for 20 min and collecting the resulting lysate which was then clarified by spinning at 10,000 rpm for 10 min.

**RESULTS**

**The Role of PKC in the Induction of MKP-1 Expression and Growth Factor-stimulated ERK Activity—** The growth factors LPA and EGF work through unique as well as shared signaling mechanisms. LPA stimulates a G-protein-coupled receptor resulting in the activation of the Gq GTPases which activate phospholipase Cβ. The products of phospholipase C activity, inositol-1,4,5-trisphosphate and sn-1,2-diradylglycerol, stimulate calcium fluxes and PKC. EGF signals through a protein-tyrosine kinase receptor in a second messenger independent manner, in which Rat-1 cells is not coupled to phospholipase C (21). Despite these differences, both LPA and EGF stimulate the Ras pathway leading to the activation of the Raf-MEK-ERK cascade and immediate early gene expression. Besides the transcription factors, c-Jun and c-Fos, the ERK phosphatase, MKP-1, is also induced by growth factors and serves to regulate the magnitude and duration of stimulated ERK activity. In addition, the phorbol ester, PMA, which activates PKC directly and mimics some of the actions of LPA, is able to activate ERK and stimulate MKP-1 expression (Fig. 1A).

In the course of studying the contribution of PKC in regulating MKP-1 expression, we noted some striking differences between two different PKC inhibitors. Pretreatment of Rat-1 cells with either Ro-31-8220 or GF109203X resulted in a complete block of PMA-induced MKP-1 expression, consistent with PKC mediating the effects of PMA. In contrast, LPA-stimulated MKP-1 expression was blocked by Ro-31-8220 but not GF109203X indicating a disparity in the actions of these two drugs. Depletion of PKC by prolonged exposure of Rat-1 cells to PMA had little effect on LPA-stimulated MKP-1 expression suggesting that although LPA can stimulate PKC, LPA makes little use of PKC for MKP-1 induction. Similarly, only Ro-31-8220 but not GF109203X is able to inhibit EGF-induced MKP-1 synthesis. This final observation is very striking in view of the fact that EGF signaling has now known PKC-dependent component in Rat-1 fibroblasts (21). Taken together, these data suggest that inhibition of MKP-1 expression by Ro-31-8220 may be due at least in part to additional PKC-independent actions of this compound (Fig. 1).

Both LPA and EGF stimulate ERK in a biphasic manner consisting of an early peak followed by a second sustained phase which persists for several hours (20, 43). It has previously been shown that inhibiting MKP-1 synthesis by treating NIH3T3 cells with cycloheximide prior to growth factor stimulation results in a potentiation of the sustained phase of ERK activity (17, 22); we have recently confirmed these results in

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3 S. J. Cook, J. Beltman, K. A. Cadwallader, M. MacMahon, and F. McCormick, manuscript in preparation.
Ro-31-8220 Inhibits Growth Factor-stimulated c-Jun Expression and Amplifies c-Jun Expression—We investigated the effects of Ro-31-8220 on other immediate early genes by looking at the expression of the transcription factors, c-Jun and c-Fos. PMA, EGF, and LPA were able to induce the expression of both c-Jun and c-Fos in Rat-1 cells, as judged by immunoblotting of whole cell lysates. In general, the c-Fos response was consistently stronger than the c-Jun response with respect to growth factors (Fig. 3) whereas stresses gave a stronger c-Jun response (Fig. 4B and data not shown). Upon stimulated expression, both c-Fos and c-Jun proteins appeared as multiple, post-translationally modified species (Fig. 3). In particular, c-Fos was apparent as a broad band which has recently been shown to contain up to four discrete immune reactive species as described by Bravo and Curran and co-workers (24); these forms of c-Fos are due to phosphorylation of specific sites (25).

As observed for MKP-1 expression, both GF109203X and Ro-31-8220 completely inhibited PMA-stimulated c-Fos expression. In contrast, GF109203X had little effect on LPA-stimulated c-Jun expression but had no effect on c-Jun expression in response to LPA. During the course of these experiments, we noted that Ro-31-8220 on its own, but not GF109203X, was able to induce the expression of c-Jun protein. This response was far greater than that observed with growth factors or with PMA. The response is rapid (within 15 min) and is sustained (observed up to 180 min) (Fig. 4B). In addition, pretreating cells with Ro-31-8220 prior to the application of growth factor serves to augment the appearance of c-Jun protein as observed by immunoblotting of time courses of c-Jun expression. 4

In separate experiments, we found that down-regulation of

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4 J. Beltman and S. J. Cook, unpublished observations.
PKC had little effect on the EGF- or LPA-stimulated c-Fos and c-Jun responses.\textsuperscript{4} Taken together, these data demonstrate that Ro-31-8220 but not GF109203X or PKC down-regulation is able to inhibit a signaling component which is required for MKP-1 and c-Fos expression but not for c-Jun expression. In addition, c-Jun expression is stimulated by Ro-31-8220, a property which may or may not be related to the inhibitory actions of this drug.

**Ro-31-8220 Is an Activator of JNK Activity**—One of the major components regulating c-Jun expression is the c-Jun\textsuperscript{2}ATF-2 complex which binds to the AP-1 site of the c-Jun promoter thereby stimulating its transcription (26, 27). This event is dependent upon the phosphorylation of c-Jun and ATF-2 by the stress activated kinases, JNK1, JNK2, and p38. We consistently observe that there is a general correlation between the strength of JNK activation and c-Jun expression in response to stress stimuli in Rat-1 cells.\textsuperscript{4} Therefore, the strong expression of c-Jun and the preliminary observation that putative alternative forms of MKP are induced by Ro-31-8220 prompted us to examine whether Ro-31-8220 might be able to activate the JNK pathway.

Rat-1 cells were treated with 5 \(\mu M\) Ro-31-8220 for increasing amounts of time and then assayed for JNK1 activity. Ro-31-8220 caused a time-dependent activation of JNK1 which rose to 8-fold by 45 min. In fact, the kinetics of JNK activation by anisomycin and Ro-31-8220 are strikingly similar (Fig. 4A). Although the magnitude of activation of JNK1 by Ro-31-8220 (25-fold \(\pm 8, n = 8, \text{data not shown}\)) is greater than for either anisomycin (10-fold \(\pm 2, n = 6\)) or Ro-31-8220 (8 \(\pm 2, n = 10\)), Ro-31-8220 activates JNK1 as well as anisomycin, a well-characterized JNK activator (Fig. 4A) (28–30). Neither PMA nor GF109203X (1.5-fold \(\pm 0.6, n = 3\)) has any effect on JNK1 activation, \(n = 3\) (Figs. 6B and 4A). Furthermore, the kinetics of JNK1 activation by Ro-31-8220 correlate well with the time course of c-Jun expression, consistent with the ability of JNK to regulate the c-Jun promoter (Fig. 4B).

The EC\textsubscript{50} for activation of JNK1 by Ro-31-8220 is 2.3 \(\pm 0.8 \mu M, n = 8\) (Fig. 5A). Significantly, these are doses which are frequently used to inhibit PKC in whole cells. The activation of JNK correlates with the dose-dependent expression of c-Jun in response to Ro-31-8220 as observed by Western blotting (Fig. 5B). These properties appear to be unique to Ro-31-8220, since GF109203X has no effect on JNK1 activity or c-Jun expression.

**Ro-31-8220 Is Not an Inhibitor of General Protein Synthesis**—Anisomycin and cycloheximide are known translational inhibitors.\textsuperscript{31} Since Ro-31-8220 could mimic the ability of anisomycin to activate JNK1 and the ability of cycloheximide to inhibit MKP-1 expression and potentiate ERK activity, we investigated whether Ro-31-8220 could be a protein synthesis inhibitor. As expected, anisomycin was able to dramatically inhibit bulk protein synthesis as determined by the incorporation of \([\text{\textsuperscript{3}H}]\text{leucine}\) into total cellular protein, as early as 30 min after application, while neither Ro-31-8220 nor GF109203X had any effect on protein synthesis even after 6 h (Table I). Thus, it appears that the ability of Ro-31-8220 to inhibit MKP-1 expression and potentiate ERK activity is not due to its acting as a translational poison.

**Ro-31-8220 Activation of JNK Is PKC-independent**—In order to determine if these novel activities of Ro-31-8220 treatment are in fact dependent upon the presence of PKC, cells were depleted of the PMA-sensitive forms of PKC by chronic treatment with PMA for 48 h prior to the application of Ro-31-8220. As determined by immunoblotting, Rat-1 fibroblasts express the \(\alpha, \delta, \epsilon,\) and \(\zeta\) forms of PKC and to a much lower level the \(\beta\) isoform. The \(\gamma\) isoform could not be detected. Of these, the \(\alpha, \delta,\) and \(\epsilon\) forms of PKC were down-regulated by chronic PMA treatment, again determined by immunoblotting (Fig. 6A). The PKC inhibitor, GF109203X, has been shown to inhibit the \(\alpha, \beta,\) and \(\gamma\) and to a much less extent \(\epsilon\) isoforms of PKC (32). Experiments similar to those described above were performed where cells were pretreated with GF109203X for 30 min prior to the application of Ro-31-8220.

Neither the down-regulation (Fig. 6, B and C) nor the bio-

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**FIG. 3.** The effect of Ro-31-8220 and GF109203X on c-Jun and c-Fos expression. Rat-1 fibroblasts were treated with vehicle, 5 \(\mu M\) Ro-31-8220, or 2.5 \(\mu M\) GF109203X for 30 min followed by stimulation with 1 \(\mu M\) PMA, 50 \(\mu M\) LPA, or 10 \(nM\) EGF for 1 h. Whole cell lysates were analyzed by immunoblotting for c-Fos and c-Jun expression. A, Ro-31-8220 inhibits PMA- and LPA-stimulated c-Fos expression (lanes 3, 6, and 9). B, Ro-31-8220 does not inhibit PMA- or LPA-stimulated c-Jun expression and actually induces c-Jun expression on its own (lanes 3, 6, and 9). In contrast, GF109203X is without effect on LPA-stimulated c-Fos or c-Jun expression, but inhibits PMA-stimulated responses (A and B, lanes 7 and 10).

**FIG. 4.** (A) Ro-31-8220 is an activator of JNK1 activity. Rat-1 fibroblasts were treated with 5 \(\mu M\) Ro-31-8220, 50 \(\mu g/ml\) anisomycin, or 2.5 \(\mu M\) GF109203X for increasing amounts of time. A, endogenous JNK1 was immunoprecipitated, an immune-complex kinase assay was performed, and \([\text{\textsuperscript{32}P}]\) incorporation into GST-c-Jun was determined by autoradiography and scintillation counting. Data shown are from a single experiment which is representative of 3 (GF109203X, 6 (anisomycin), or 10 (Ro-31-8220) similar experiments. B, the stimulation of c-Jun expression in response to Ro-31-8220 was determined by immunoblotting of whole cell lysates.
chemical inhibition of PKC with GF109203X had any effect on the ability of Ro-31-8220 to activate JNK or stimulate c-Jun expression. This suggests that the ability of Ro-31-8220 to activate stress pathways is independent of PKC although we cannot presently rule out the possibility that Ro-31-8220 acts on a subset of PKC isozymes that are not down-regulated by PMA or inhibited by GF109203X. This possibility seems unlikely, however, since the specificity of Ro-31-8220 and GF109203X overlap significantly with regard to isozyme specificity.

**DISCUSSION**

Protein kinase C is a multifunctional protein kinase family whose members exhibit distinct properties including sensitivity to calcium and the phorbol ester family of tumor promoters. Various PKC isozymes have been implicated in diverse cellular processes including inflammation, mitogenesis, and differentiation. Activation of PKC is sufficient to activate Raf-MEK-ERK cascade and expression of immediate early genes and likely represents a cooperative mitogenic signal. It is therefore of considerable interest to be able to antagonize the effect of PKC either as a family or as individual isozymes.

The bisindolylmaleimides, Ro-31-8220 and GF109203X, are two structurally related staurosporine analogs which act as ATP-competitive inhibitors of PKC and have been used extensively for studying the role of PKC in cell signaling (3). Several reports have pointed out differential effects between various bisindolylmaleimides and in particular between GF109203X and Ro-31-8220. The initial characterization of 5 bisindolylmaleimide staurosporine analogs showed that they all displayed a slight selectivity for PKCα over other PKC isofoms tested (33). Ro-31-8220 displayed the greatest selectivity of the tested analogs; the IC₅₀ for inhibition of PKCα (5 nM, *in vitro*) was...
approximately 5-fold less than that for PKCβ, -γ, -ε, -ζ. The effect of bisindolylmaleimide PKC inhibitors on PKCδ and PKCζ was not addressed in this study. In vivo, the selectivity of Ro-31-8220 and GF109203X was addressed in αT3–1 gonadotrophs expressing PKCa, -ε, and -ζ. In that study, Ro-31-8220 was shown to be a selective inhibitor of calcium-, diterpine-, and phorbol ester-activable PKC isozymes α, ε, and ζ activity as of activated PKC activity (34). Other studies have addressed the selectivity of staurosporine and its analogs to inhibit soluble PKC and membrane-associated PKC. Ro-31-8220, in particular, showed a striking selectivity for membrane-associated PKC, while GF109203X inhibited the soluble and membrane-associated forms of PKC to the same extent (35, 36). Although all of these studies suggest that GF109203X is less selective for particular PKC isozymes than Ro-31-8220, the molecular reasons for these observations remain speculative. This report reveals several unique and novel properties of Ro-31-8220.

The ability of Ro-31-8220 to inhibit growth factor-stimulated expression of MKP-1 and c-Fos suggests that this drug targets a signaling component, perhaps a PKC-related serine/threonine kinase, common to both these pathways which is absolutely required for induction of gene expression. Activation of PKC by PMA is sufficient to induce both MKP-1 and c-Fos expression indicating that PMA-sensitive isofoms can at least impinge on the signaling pathways regulating the expression of these two genes. However, there is little evidence that the conventional PKC isozymes are necessary for growth factor-stimulated expression of these genes. Inhibition of PKC by Ro-31-8220 or GF109203X or the depletion of PKC by chronic PMA treatment inhibited PMA-stimulated MKP-1 and c-Fos expression, consistent with all three of these treatments antagonizing PMA-sensitive forms of PKC. However, GF109203X or PKC depletion had little or no effect on LPA- or EGF-stimulated expression of MKP-1 or c-Fos expression, yet Ro-31-8220 inhibited both these responses. In the case of MKP-1, this inhibition of expression resulted in the potentiation of the sustained phase of LPA- and EGF-stimulated ERK1 activity. This result is analogous to that observed when MKP-1 expression is inhibited by cycloheximide, although we have demonstrated that, unlike cycloheximide, Ro-31-8220 is not a protein synthesis inhibitor. GF109203X did not potentiate LPA- or EGF-stimulated ERK activity, suggesting that it is not a PKC-mediated effect but rather a specific effect of Ro-31-8220. Ro-31-8220 could perhaps act by inhibiting a PKC-related kinase, such as the novel recently described PKC-related kinases, PRK-1 or PRK (37–39). However, we cannot presently rule out the possibility that Ro-31-8220 may be an inhibitor of a PMA- and GF109203X-insensitive PKC isozyme.

These effects of Ro-31-8220 point to an extension of its previously defined pharmacological actions. However the ability of Ro-31-8220 but not GF109203X to stimulate JNK1 and induce c-Jun expression point to an entirely new set of pharmacological properties. The ability of Ro-31-8220 to stimulate JNK1 and induce c-Jun expression appears to be dissociable form PKC inhibition by several criteria. First, PMA is a potent activator of PKC but is unable to stimulate JNK1 activity. Secondly, neither the prior down-regulation of PKCa, - δ, and -ε nor the inhibition of PKCa, - β, and -γ by GF109203X has any effect on the ability of Ro-31-8220 to activate JNK1 or stimulate c-Jun expression. Although we have not addressed the role of PKCζ in JNK activation directly in this study, when a kinase dead or activated mutant of PKCζ was co-expressed in COS cells with SEK1 and JNK1, it was determined that this particular PKC isozyme played no role in the activation of JNK1 (12).

All of these points indicate that the presence of PKC protein and PKC catalytic activity are unrelated to the ability of Ro-31-8220 to stimulate stress signaling pathways.

This study showing that Ro-31-8220 activates JNK and stimulates c-Jun expression at concentrations commonly used in whole cells to inhibit PKC negate its further use as a selective PKC inhibitor in whole cells. The EC50 for JNK activation is 2.3 μM. When Rat-1 cells are incubated in 5 μM Ro-31-8220, an 8-fold activation of JNK1 is observed and c-Jun expression is strongly induced. These concentrations of Ro-31-8220 have frequently been used to assess the role of PKC in cellular responses (40–42). Although the unique properties of Ro-31-8220 described in this report suggest that the conclusions of some of these earlier reports may need to be reinterpreted, they may also point out additional roles for the stress-activated signaling cascades. The ability of Ro-31-8220, an ATP competitive serine/threonine protein kinase inhibitor to stimulate stress-activated pathways raises the possibility that inhibition of some protein kinases may be perceived by the cell as stressful. If this is the case, this involves the inhibition of a subset of protein kinases since neither the inhibition of PKC signaling by GF109203X (Fig. 4) or PKC down-regulation (Fig. 6) nor the inhibition of the ERK cascade by the MEK inhibitor PD98059h has any effect on JNK1 activity. It will be of interest to determine what the target of Ro-31-8220 is and whether that protein may play a role in signaling cascades regulating cell cycle progression or arrest.

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The Selective Protein Kinase C Inhibitor, Ro-31-8220, Inhibits Mitogen-activated Protein Kinase Phosphatase-1 (MKP-1) Expression, Induces c-Jun Expression, and Activates Jun N-terminal Kinase
Jerlyn Beltman, Frank McCormick and Simon J. Cook

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