

## Paradoxical activation of Raf by a novel Raf inhibitor

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**Background:** Raf is a proto-oncogene that is activated in response to growth factors or phorbol esters, and is thought to activate MAP kinase kinase-1 (MKK1) and hence the classical MAP kinase (MAPK) cascade.

**Results:** The compound ZM 336372 is identified as a potent and specific inhibitor of Raf isoforms *in vitro*. Paradoxically, exposure of cells to ZM 336372 induces >100-fold activation of c-Raf (measured in the absence of compound), but without triggering any activation of MKK1 or p42 MAPK/ERK2. The ZM 336372-induced activation of c-Raf occurs without any increase in the GTP-loading of Ras and is not prevented by inhibition of the MAPK cascade, protein kinase C or phosphatidylinositol 3-kinase. ZM 336372 does not prevent growth factor or phorbol ester induced activation of MKK1 or p42 MAPK/ERK2, or reverse the phenotype of Ras- or Raf-transformed cell lines. The only other protein kinase inhibited by ZM 336372 out of 20 tested was SAPK2/p38. Although ZM 336372 is structurally unrelated to SB 203580, a potent inhibitor of SAPK2/p38, the mutation of Thr106→Met made SAPK2/p38 insensitive to ZM 336372 as well as to SB 203580.

**Conclusions:** Raf appears to suppress its own activation by a novel feedback loop, such that inhibition is always counterbalanced by reactivation. These observations imply that some agonists reported to trigger the cellular activation of c-Raf might actually be inhibitors of this enzyme, and that compounds which inhibit the kinase activity of Raf might not be useful as anticancer drugs. The binding sites for ZM 336372 and SB 203580 on Raf and SAPK2/p38 are likely to overlap.

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## Introduction

The sustained activation of the classical mitogen-activated protein (MAP) kinase cascade is believed to play a critical role in inducing the proliferation of some cells and the differentiation of others [1]. In this pathway, growth factors trigger the dimerisation and activation of their receptors leading to transphosphorylation of the receptors at several tyrosine residues [2]. These phosphotyrosines then interact with proteins containing SH2 domains, two of which are the 'adaptors' Grb2 and Shc [3–5]. Grb2 is complexed to the guanine nucleotide exchange factor mSos that activates Ras [6,7]. Thus the binding of Grb2 to phosphorylated receptors (and/or to the tyrosine phosphorylated form of Shc, which is bound to these receptors) recruits mSos from the cytosol to the plasma membrane where it converts Ras from the inactive GDP-bound form to the active GTP-bound state [8].

Ras is associated with the plasma membrane by virtue of lipid modifications at its carboxyl terminus and its membrane localisation is essential for activation by growth factors [9]. GTP-Ras interacts with the protein kinase c-Raf, localising the latter to the plasma membrane

[10–12] where it becomes activated by a mechanism(s) that is not yet fully understood [13]. The activation of c-Raf is then thought to induce the sequential activation of MAP kinase kinase-1 (MKK1) and the MAP kinases p44 MAPK and p42 MAPK (also known as ERK1 and ERK2) that, in turn, activate other protein kinases, such as MAPK-activated protein kinase-1 (MAPKAP-K1, also known as Rsk). When activation of the MAPKs is sustained, they translocate from the cytosol to the nucleus to regulate transcriptional events that are thought to be critical for proliferation or differentiation [14–17].

Inappropriate activation of the MAP kinase cascade can lead to the uncontrolled proliferation or differentiation of cells, and the mutation or overexpression of components of the MAP kinase cascade is a common feature of many cancer cells. For example, the epidermal growth factor (EGF) receptor is overexpressed or mutated in many cancers of epithelial origin, the platelet-derived growth factor (PDGF) receptor in cancers of mesenchymal or glial origin and the nerve growth factor (NGF) receptor in prostate cancer and prostate hyperplasia (reviewed in [18]). Ras is mutated to an activated, oncogenic form in a high

proportion of human cancers [19], c-Raf is the cellular homologue of a viral oncogene [20], and the overexpression and hyperactivation of MAP kinase in a number of breast cancers has been reported [21]. For these reasons, specific inhibitors of different components of these pathways might have therapeutic potential as anticancer agents. Indeed, drugs that inhibit the EGF receptor and the PDGF receptor are undergoing human clinical trials, and farnesyl transferase inhibitors have been developed that prevent the activation of Ras. In the MAP kinase cascade itself, a compound (PD 98059) has been identified that interacts with MKK1 and prevents its activation by c-Raf [22]. This compound can reverse the transformed phenotype of several Ras-transformed cell lines [23].

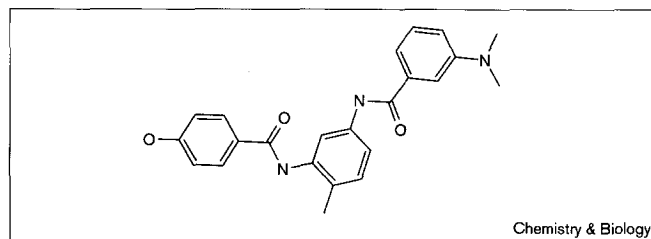
Here, we describe a compound (ZM 336372) that is a potent inhibitor of the protein kinase c-Raf *in vitro*. Paradoxically, however, incubation of mammalian cells with this compound induces an enormous activation of c-Raf and the B-Raf isoform (measured in the absence of the drug), suggesting that a feedback control loop exists by which Raf isoforms suppress their own activation. This unexpected finding may explain why ZM 336372 does not reverse the phenotype of Ras-transformed cell lines, and suggests that inhibition of the kinase activity of Raf might not be a good approach for the development of an anti-cancer drug.

## Results

### Identification of ZM 336372 as an inhibitor of c-Raf

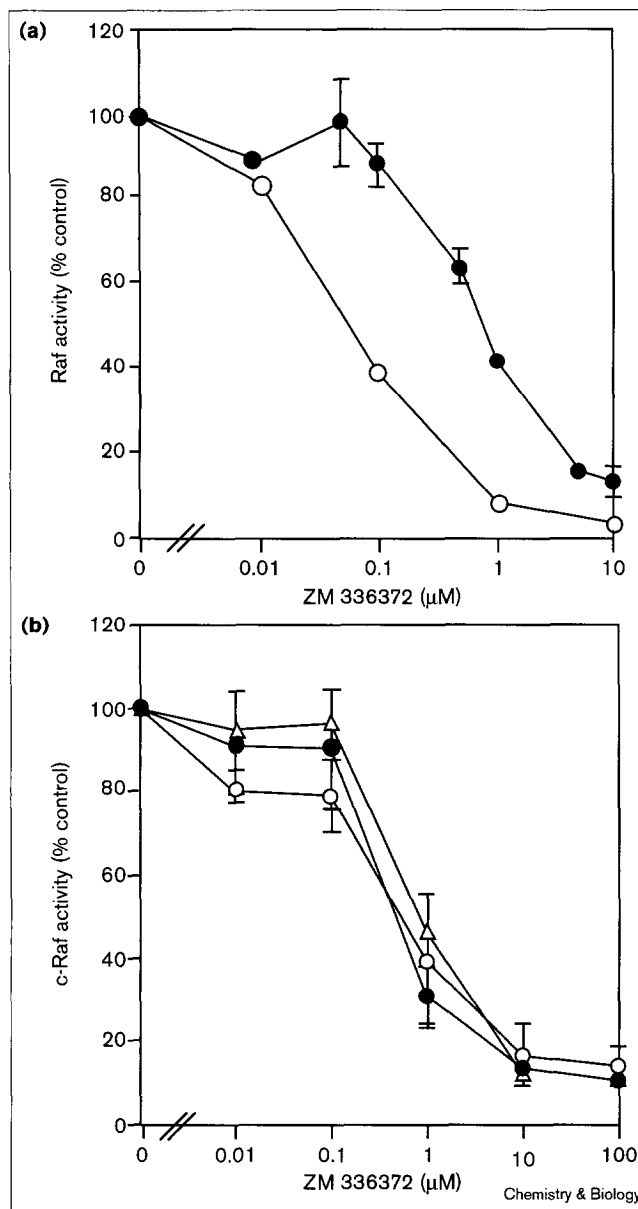
ZM 336372 (Figure 1) was identified by screening a compound library with a single step coupled *in vitro* kinase cascade assay that measured the phosphorylation of myelin basic protein (MBP) in the presence of c-Raf, GST-MKK1 and GST-p42 MAPK [24]. The c-Raf had been activated by cotransfection in Sf9 insect cells with v-Ras and Lck and was added without purification. Because of the sensitivity of the assay, the Sf9 cell extracts were used at 50,000-fold dilution or greater [24]. Control Sf9 lysates were used to demonstrate that incorporation of radioactivity into MBP was dependent on the activity of c-Raf. In order to determine the molecular

Figure 1



The chemical structure of ZM 336372. ZM 336372 is N-[5-(3-dimethylaminobenzamido)-2-methylphenyl]-4-hydroxybenzamide [35].

Figure 2



The effect of ZM 336372 on c-Raf activity *in vitro*. (a) Human c-Raf (open circles) or human B-Raf (closed circles) were activated in Sf9 cells by cotransfection from baculovirus vectors containing DNA encoding v-Ras and Lck in the absence of ZM 336372. The cell lysates were then assayed for c-Raf activity in the absence or presence of the indicated concentrations of ZM 336372. (b) c-Raf was activated in mouse Swiss 3T3 cells by incubation for 2 min with 100 ng/ml EGF or 0.4 μg/ml PMA (open circles), in human 293 cells by incubation for 2 min with 100 ng/ml EGF or 0.4 μg/ml PMA (closed circles) or in Sf9 cells (open triangles) by coexpression with DNA encoding v-Ras and Lck. ZM 336372 was excluded from these incubations. The c-Raf activity was then assayed in the absence or presence of the indicated concentrations of ZM 336372 after its immunoprecipitation from the cell lysates. The data obtained after stimulation with EGF or phorbol esters were combined because the  $IC_{50}$  values for c-Raf were identical. The results are presented relative to control incubations in which ZM 336372 was omitted (mean  $\pm$  SEM for three separate experiments).

target of ZM 336372, each kinase was subsequently assayed individually in the presence of the inhibitor. The inhibitor was found to have no discernible effect on either the ability of MKK1 to phosphorylate p42 MAPK or the ability of p42 MAPK to phosphorylate MBP, even at concentrations as high as 100  $\mu$ M. Similarly, the compound did not inhibit a coupled *in vitro* kinase cascade assay involving MKK1, p42 MAPK and MBP, in which MKK1 had previously been activated by c-Raf or mutated to a constitutively active form by changing Ser218 and Ser222 to glutamic acid. These data unambiguously identified c-Raf as the only kinase in the primary coupled kinase screen that was inhibited by the compound over a 1000-fold concentration range.

#### Selectivity of ZM 336372 as a Raf inhibitor

ZM 336372 is a potent inhibitor of human c-Raf. The  $IC_{50}$  value was 0.07  $\mu$ M in the standard assay (Figure 2a), which contains 0.1 mM ATP. The  $IC_{50}$  decreased to 0.01  $\mu$ M at 0.025 mM ATP and increased to 0.9  $\mu$ M at 2.5 mM ATP (data not shown) indicating that ZM 336372 is a competitive inhibitor with respect to ATP. The compound inhibited

c-Raf tenfold more potently than B-Raf (Figure 2a) and did not inhibit 17 of 19 other protein kinases tested, even at 50  $\mu$ M (Table 1). The two exceptions were SAPK2a/p38 $\alpha$  and SAPK2b/p38 $\beta$  (Table 1), both of which were inhibited by ZM 336372 with an  $IC_{50}$  of 2  $\mu$ M under the assay conditions used. The effect of ZM 336372 on SAPK2a/p38 $\alpha$  and SAPK2b/p38 $\beta$  activity is detailed at the end of the Results section.

In the experiments shown in Figure 2a, c-Raf and B-Raf were assayed directly in Sf9 cell lysates. If c-Raf was assayed after its immunoprecipitation from the lysates of Sf9 cells, human 293 cells or mouse Swiss 3T3 cells, however, the  $IC_{50}$  values were tenfold higher (Figure 2b), indicating that interaction of c-Raf with the antibody decreases its affinity for the drug. The  $IC_{50}$  values were similar whether the 293 cells or the Swiss 3T3 cells were stimulated with EGF or phorbol 12,13 myristate acetate (PMA; see the legend to Figure 2).

#### Failure of ZM 336372 to prevent the activation of MKK1 or p42 MAPK in mammalian cells

As c-Raf and B-Raf are believed to lie at the head of the classical MAP kinase cascade, we next examined whether ZM 336372 would prevent the activation of MKK1 (an immediate downstream target of these enzymes) and p42 MAPK in cells. Surprisingly, ZM 336372 (even at 10  $\mu$ M) only suppressed the EGF-induced activation of MKK1 by 37%, and had no effect on the activation of p42 MAPK (Figure 3). ZM 336372

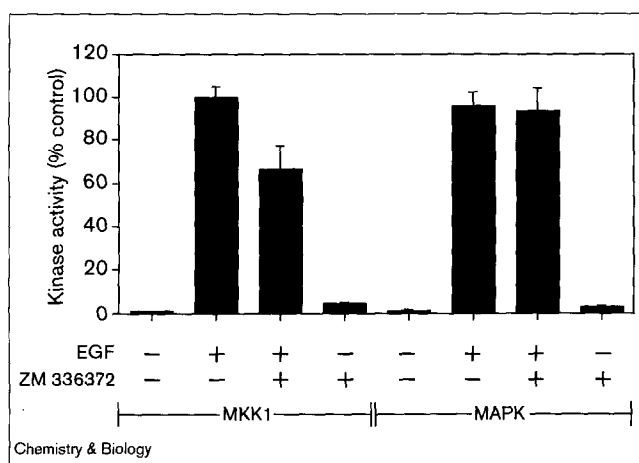
**Table 1**

**Effect of 50  $\mu$ M ZM 336372 on the activities on protein kinases *in vitro*.**

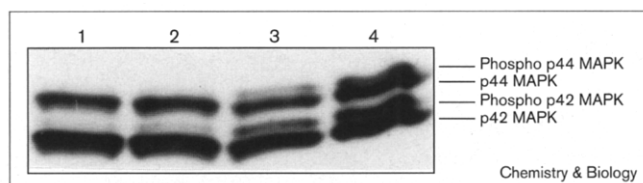
Protein kinase	Activity (% control)
Protein kinase A	56 $\pm$ 2
Protein kinase B $\alpha$	92 $\pm$ 2
Protein kinase C	92 $\pm$ 2
AMPK	102 $\pm$ 6
MAPKAP kinase 1B	86 $\pm$ 6
MAPKAP kinase 2	93 $\pm$ 7
MAPKAP kinase 3	89 $\pm$ 2
p70 S6 kinase	99 $\pm$ 1
CK2	111 $\pm$ 2
p42 MAPK	105 $\pm$ 5
MKK1	99 $\pm$ 2
GSK3 $\beta$	103 $\pm$ 2
MKK4	86 $\pm$ 8
SAPK1/JNK	102 $\pm$ 1
SAPK3	102 $\pm$ 3
SAPK4	92 $\pm$ 5
Cyclin B/CDK1	106 $\pm$ 4
SAPK2a	3 $\pm$ 2
SAPK2b	12 $\pm$ 1

The protein kinases were incubated for 10 min at 4°C in the presence or absence of 50  $\mu$ M ZM 336372 and then assayed for activity at 30°C in the presence of 0.1 mM ATP. Activities are given as a mean  $\pm$  SEM relative to control incubations in which the inhibitor was omitted. AMPK, AMP-activated protein kinase; MAPK, mitogen-activated protein kinase; MAPKAP, MAPK-activated protein; GSK3, glycogen synthase kinase-3; MKK, MAPK kinase; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; CK2, casein kinase-2; CDK1, cyclin-dependent protein kinase-1, also called cdc2.

**Figure 3**



Effect of ZM 336372 on the activation of MKK1 and p42 MAPK in Swiss 3T3 cells. Confluent Swiss 3T3 cells were incubated for 18 h in Dulbecco's modified Eagles medium (DMEM) containing 0.5% fetal calf serum (FCS) and then for 60 min with or without 10  $\mu$ M ZM 336372 followed by stimulation for 5 min with 100 ng/ml EGF. The cells were lysed and MKK1 and p42 MAPK activities measured after immunoprecipitation. The results are presented as mean  $\pm$  SEM for three separate experiments.

**Figure 4**

Effect of ZM 336372 on MAPK phosphorylation in HCT 116 cells. The figure shows a MAPK western blot of lysates from cells treated for 16 h with: lane 1, 10  $\mu$ M PD 98059; lane 2, 50  $\mu$ M PD 98059; lane 3, no compound control; lane 4, 10  $\mu$ M ZM 336372 in the presence of 10% FCS.

also failed to affect the activation of p42 MAPK induced by stimulating Swiss 3T3 cells with PMA or L6 cells with insulin-like growth factor-I (IGF-I; data not shown). Consistent with these observations, and unlike PD 98059 [23], ZM 336372 (10  $\mu$ M) also failed to inhibit the constitutive activation of p44 MAPK or p42 MAPK seen in the human colon tumour cell line HCT116 (Figure 4) and H-ras transformed NIH3T3 cells (data not shown) and did not affect either the proliferation or morphology of NIH3T3 cells transformed by either overexpression of H-ras or constitutively activated c-Raf (Table 2). Moreover, unlike PD 98059, ZM 336372 was similarly unable to affect proliferation of untransformed NIH3T3 cells that had been stimulated by various mitogens (Table 3).

#### Paradoxical activation of c-Raf by ZM 336372 in mammalian cells

In order to investigate why ZM 336372 did not prevent the activation of MKK1 or p42 MAPK, we exposed Swiss 3T3 cells to ZM 336372 and then measured c-Raf activity in the absence of ZM 336372 after its immunoprecipitation from the cell lysates. These experiments revealed that exposure to ZM 336372 induced a remarkable activation of c-Raf that increased several hundredfold after 60 min and

**Table 2**

**Effect of ZM 336372 on the growth and morphology of transformed NIH3T3 cells.**

	10 $\mu$ M ZM 336672 (% control)	50 $\mu$ M PD 98059 (% control)
Monolayer growth		
H-ras 3T3	46	8
Raf 3T3	125	7
Morphology		
H-ras 3T3	No effect	Fully reverted
Raf 3T3	No effect	Fully reverted

NIH3T3 cells transfected with H-ras or the catalytic domain of c-Raf were cultured for 6 days in 5% charcoal stripped serum in the presence of compound before scoring the effect on proliferation (cell number presented as percentage of untreated cells) and morphology, (scored relative to untransformed NIH3T3 cells).

**Table 3**

**Effect of ZM 336372 on the proliferation of NIH3T3 cells stimulated with various mitogens.**

Stimulus	10 $\mu$ M ZM 336372 (% control)	20 $\mu$ M PD 98059 (% control)
PDGF	45	24
FGF	81	13
LPA	99	1
EGF	68	29
Insulin	90	29
Basal growth	115	-20

NIH3T3 cells were cultured for 6 days in charcoal stripped medium, in the presence of 10 ng/ml PDGF, 10 ng/ml fibroblast growth factor (FGF), 10  $\mu$ l/ml lysophosphatidic acid (LPA), 10 ng/ml EGF, 10  $\mu$ g/ml insulin or media alone in order to determine the effect of compounds on growth factor stimulated and basal cell growth. 0.02 ml of 2.5 mM phenazine methosulphate was added to 1 ml of 1 mg/ml XTT (Sigma) and 0.125 ml of the mixture added to the culture medium of each dish of cells. After incubation for 2 h at 37°C, the absorbance at 450 nm was measured. Values are presented as percent of that without compound.

was sustained for at least 3 h (Figure 5a). The activation was much higher than that attained after stimulation with EGF (Figure 5a), which is the most potent inducer of c-Raf activation in Swiss 3T3 cells [22]. However, ZM 336372 did not itself induce any activation of p42 MAPK (Figures 3,5a). In contrast, EGF potently induced p42 MAPK activation irrespective of whether the Swiss 3T3 cells had been preincubated with ZM 336372 (Figure 3). This was despite the fact that, after incubation of the cells for 1 h with ZM 336372, subsequent stimulation with EGF only increased the level of c-Raf activity by 20–30% (data not shown).

Similar results were obtained in several other cells. For example, incubation of monkey COS1 cells or human 293 cells for 1 h with 10  $\mu$ M ZM 336372 also increased c-Raf activity over 100-fold (data not shown) and the analogous experiment in PC12 cells increased B-Raf activity 100-fold (data not shown). Under these conditions, ZM 336372 did not itself induce any activation of p42 MAPK in PC12 cells, consistent with the results in other cells.

In order to further demonstrate the effect of ZM 336372 on c-Raf activity we preincubated NIH3T3 cells with compound for several hours and then observed the effect of removing ZM 336372 on MKK1 and p42 MAPK. Removal of the inhibitor triggered an activation of MKK1 and p42 MAPK (Figure 5b), implying that the cell does indeed contain high specific activity c-Raf that is prevented from activating its downstream substrate MKK1 by the inhibitor. No activation of MKK1 and p42 MAPK occurred in control experiments carried out in parallel in which ZM 336372 was omitted (data not shown).

### Mechanism of activation of c-Raf by ZM 336372

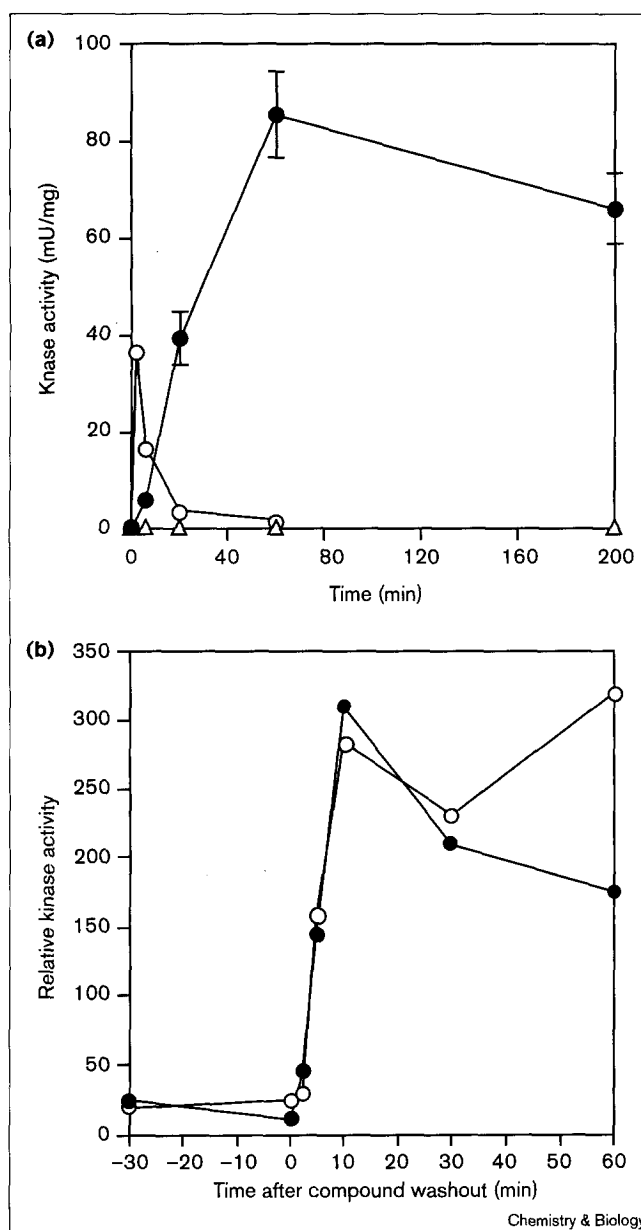
The compound PD 98059, which binds to MKK1 and prevents its activation by c-Raf (see the Introduction section), also stimulates the basal activity of c-Raf several-fold, as well as the rate and extent of c-Raf activation induced by PDGF [22]. These findings suggest that activation of the classical MAP kinase pathway suppresses the activation of c-Raf. Incubation of Swiss 3T3 cells with 50  $\mu$ M PD 98059, prevents the EGF- or PMA-induced activation of MAPKAP-K1 (Figure 6b), the most downstream component of the MAP kinase cascade. However, the activation of c-Raf induced by ZM 336372 was only reduced by 40–50% (Figure 6a). The activation of c-Raf by ZM 336372 is therefore largely independent of the activation of the MAP kinase cascade.

The activation of c-Raf by different agonists can result from the stimulation of several signal transduction pathways, including the activation of protein kinase C [13] or phosphatidylinositide (PtdIns) 3-kinase [25]. The activation of c-Raf induced by ZM 336372, however, does not appear to occur via either of these pathways. Thus c-Raf activation was unaffected by Ro 318220 (Figure 6a), an inhibitor of protein kinase C and other protein kinases [26], under conditions where it suppressed the PMA-induced activation of p42 MAPK by 85% (data not shown). Similarly, an inhibitor of PtdIns 3-kinase (wortmannin), did not affect the activation of c-Raf by ZM 336372 (Figure 6a) under conditions where the IGF-1-induced activation of protein kinase B in Swiss 3T3 cells was suppressed by 95% (data not shown).

The activation of c-Raf by growth factors, such as EGF, is dependent on the activation of Ras. In unstimulated Swiss 3T3 cells the percentage of GTP-Ras relative to GDP-Ras + GTP-Ras was  $10.7 \pm 2.6\%$ , which increased to  $44 \pm 2.4\%$  after stimulation for 2 min with EGF (Figure 7). After incubation for 60 min with ZM 336372 (at which time c-Raf activation is maximal; Figure 5a), however, the proportion of GTP-Ras had not increased at all ( $9.5 \pm 1.8\%$ ) (Figure 7). Thus the activation of c-Raf by ZM 336372 does not result from an increase in the GTP-loading of Ras. In addition, pretreatment of Swiss 3T3 cells for 120 min with ZM 336372, followed by washout of the compound as described in the legend to Figure 5b, did not cause any activation of Ras, despite the induced activation of the MAP kinase cascade (data not shown).

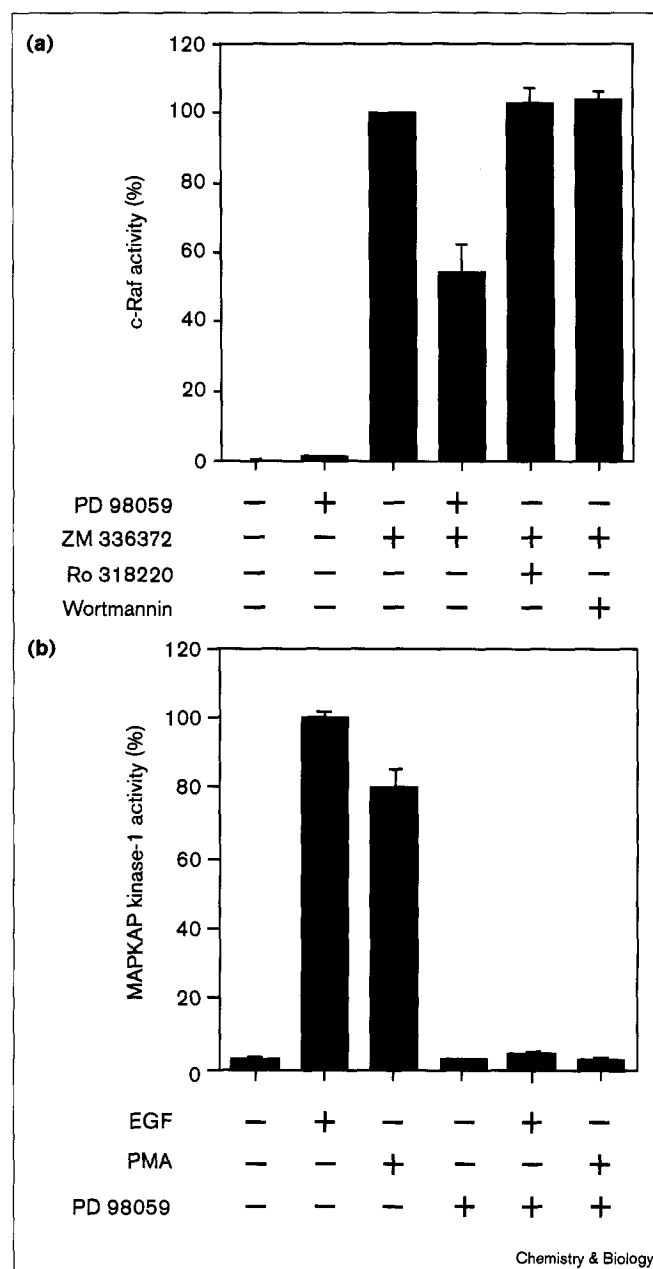
Prolonged mitogenic stimulation leads to the hyperphosphorylation of c-Raf that can be visualised by a reduction in its electrophoretic mobility, and prevented by incubating the cells with PD 98059 [22]. Pretreatment of Swiss 3T3 cells for 6–200 min with ZM 336372 did not change the electrophoretic mobility of c-Raf, as shown by immunoblotting (data not shown). These experiments also established that the level of c-Raf protein did not change over the duration of the experiment.

**Figure 5**



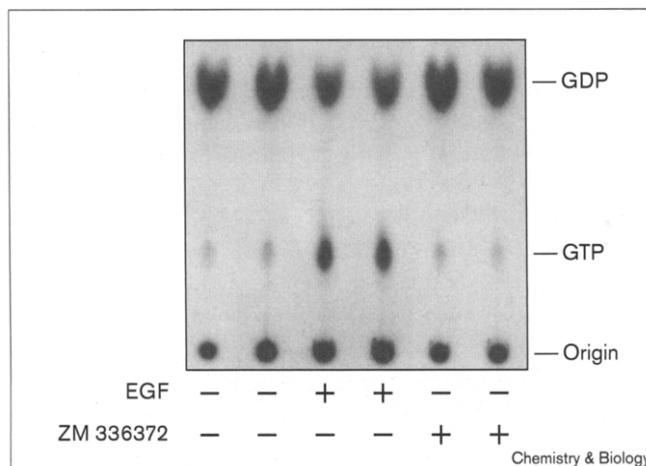
Effect of ZM 336372 addition or removal on the activation of c-Raf and the MAPK cascade. (a) Confluent Swiss 3T3 cells were incubated for 18 h in DMEM containing 0.5% FCS and then treated for the times indicated with 10  $\mu$ M ZM 336372 (closed circles) or 100 ng/ml EGF (open circles) and the activity of c-Raf measured in the absence of ZM 336372 after its immunoprecipitation from the cell lysates. p42 MAPK was also immunoprecipitated from cell lysates following treatment with 10  $\mu$ M ZM 336372 and its kinase activity assayed (open triangles). The results are presented as mean  $\pm$  SEM for three separate experiments. (b) NIH3T3 cells were incubated for 120 min in the presence of 10  $\mu$ M ZM 336372 and 0.5% FCS before washing the cells three times in Hanks buffered salt solution and replacing with fresh medium without compound. Cells were lysed at various times after compound washout and assayed for MKK (open circles) and MAPK (closed circles) activities. Results shown for each time point are based on raw data relative to each other for each kinase assayed.

Figure 6



Effect of PD 98059, wortmannin and Ro 318220 on the activation of c-Raf by ZM 336372. **(a)** Confluent Swiss 3T3 cells were incubated in DMEM containing 0.5% FCS for 18 h and then pretreated with (+) or without (-) 50  $\mu$ M PD 98059 (60 min), 100 nM wortmannin (10 min) or 0.3  $\mu$ M Ro 318220 (60 min) prior to incubation for 60 min with 10  $\mu$ M ZM 336372 in the continued presence of these compounds. The activity of c-Raf was measured after immunoprecipitation from the cell lysates and the results are presented as a percentage of the activity measured after incubation for 60 min with ZM 336372 in the absence of any other compound. **(b)** Swiss 3T3 cells were cultured as for (a) except that cells were stimulated with 100 ng/ml EGF or 0.4  $\mu$ g/ml PMA with (+) or without (-) 60 min pretreatment with 50  $\mu$ M PD 98059. The activity of MAPKAP kinase-1 was measured after immunoprecipitation from the cell lysates and the results are presented as a percentage of the activity measured after incubation for 10 min with EGF.

Figure 7



Lack of effect of ZM 336372 on the GTP-loading of Ras in Swiss 3T3 cells. Cells were labelled as described in the Materials and methods section before stimulation with 100 ng/ml EGF (2 min) or 10  $\mu$ M ZM 336372 (60 min). Ras was immunoprecipitated from the cell lysates and the bound guanine nucleotides eluted and separated using thin-layer chromatography. The figure shows an autoradiograph of a representative experiment. The quantitation of these experiments is presented in the text.

#### Mechanism of inhibition of c-Raf and SAPK2/p38 by ZM 336372

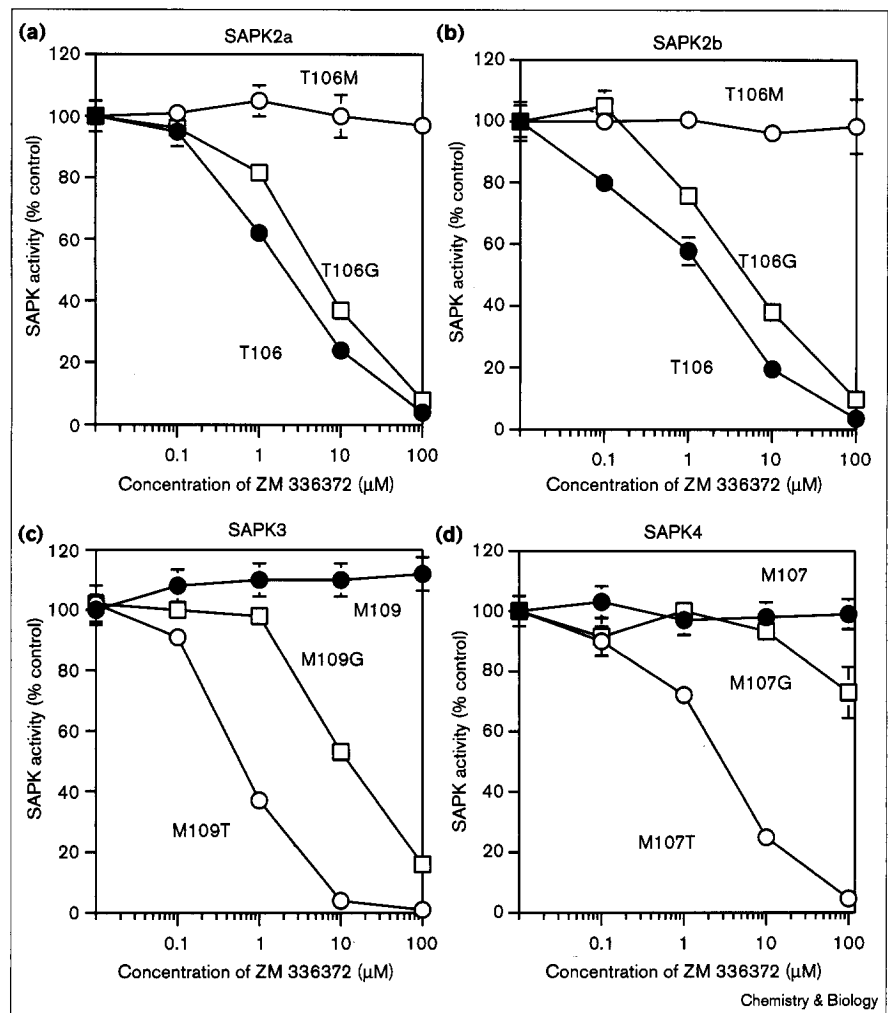
The finding that ZM 336372 inhibits SAPK2 $\alpha$ /p38 $\alpha$  and the closely related homologue SAPK2 $\beta$ /p38 $\beta$  (collectively termed SAPK2/p38), as well as Raf (Table 1), was intriguing, because we have reported that SB 203580 (a relatively specific inhibitor of SAPK2/p38) also inhibits c-Raf [27]. Although the structures of ZM 336372 (Figure 1) and SB 203580 are completely different, these observations raised the possibility that the binding sites for ZM 336372 and SB 203580 were identical or at least overlapping.

A residue that is critical for the interaction of SB 203580 with SAPK2 $\alpha$ /p38 $\alpha$  is Thr106, because the 4-fluorophenyl ring of this drug cannot be accommodated if a sidechain larger than threonine is present at this position. Thus we [28] and others [29,30] have shown that SAPK2/p38 becomes insensitive to SB 203580 if Thr106 is mutated to a residue with a more bulky sidechain, such as methionine or glutamine. The mutation of Thr106 $\rightarrow$ Met also abolished sensitivity of SAPK2 $\alpha$ /p38 $\alpha$  or SAPK2 $\beta$ /p38 $\beta$  to ZM 336372 (Figure 8a,b).

Other MAP kinase family members are insensitive to SB 203580 because the position equivalent to Thr106 of SAPK2 $\alpha$ /p38 $\alpha$  is occupied by methionine or glutamine, but these enzymes can be made sensitive to SB 203580 by mutating this residue to threonine [28,30,31]. The results presented in Figure 8c,d showed that two other MAP kinase family members, SAPK3 and SAPK4, could

**Figure 8**

Inhibition of wild-type and mutant (a) SAPK2a/p38 $\alpha$ , (b) SAPK2b/p38 $\beta$ 2, (c) SAPK3 and (d) SAPK4 by ZM 336372. The effect of the drug on the wild-type enzymes is shown by the closed circles and its effect on mutant enzymes by open symbols. Mutations are designated using the single-letter amino acid code. SAPK2a/p38 $\alpha$  and SAPK2b/p38 $\beta$ 2 are inhibited by ZM 336372 with IC<sub>50</sub> values of 2  $\mu$ M. Mutation of Thr106 $\rightarrow$ Met in SAPK2a/p38 $\alpha$  and SAPK2b/p38 $\beta$ 2 abolishes sensitivity to ZM 336372. SAPK3 and SAPK4 are resistant to ZM 336372 because they have methionine residues at positions 109 and 107 respectively. Mutation of these residues to threonine makes these enzymes sensitive to the drug. Mutation to glycine of the amino acid at the position equivalent to Thr106 leads to an increase in the IC<sub>50</sub> values in all four protein kinases. All mutants were generated, expressed, purified and activated as in [28].



Chemistry &amp; Biology

be made sensitive to ZM 336372 by the same mutation. In the case of SB 203580, MAP kinases can be made even more sensitive to the drug by substituting even smaller residues, such as serine, alanine and glycine [28]. In contrast, however, mutation to these residues made SAPK2/p38, SAPK3 and SAPK4 less sensitive to ZM 336372 than mutation to threonine (Figure 8, Table 4).

## Discussion

In this paper we have identified a potent and specific inhibitor of c-Raf (ZM 336372) that shows a tenfold selectivity over B-Raf (Figure 2a), a 30-fold selectivity over SAPK2/p38 and does not inhibit 17 other protein kinases tested (Table 1). Paradoxically, however, incubation of cells with ZM 336372 induces a huge activation of c-Raf (measured in the absence of this drug) that, in 3T3 cells, is twice as great as that induced by the most potent agonist described previously (EGF). The simplest interpretation of this result is that cells have a feedback loop

by which Raf suppresses its own activation, so that any inhibition of Raf is rapidly counterbalanced by its reactivation. This explanation is supported by the observation that ZM 336372 does not by itself induce the activation of MKK1 or p42 MAPK, yet removing it causes an activation of MKK1 and p42 MAPK (Figure 5b). This implies that the cell does indeed contain high specific activity c-Raf that is prevented from activating its downstream substrate MKK1 because of the presence of the inhibitor. The molecular mechanism by which this feedback loop operates appears to be novel, because it is not affected by inhibitors of protein kinase C, PtdIns 3-kinase or the MAP kinase cascade, and does not involve any change in the GTP loading of Ras. Our findings also raise the possibility that some substances reported to trigger the activation of c-Raf in cells might really be inhibitors of this enzyme. Nevertheless, it cannot yet be excluded that ZM 336372 exerts its effect on c-Raf more indirectly by activating or inhibiting another unknown target allied to c-Raf activation.

Table 4

**Effect of mutating Met109 on the sensitivity of SAPK3 to ZM 336372 and SB 203580.**

Residue at 109	ZM 336372 IC <sub>50</sub> (μM)	SB 203580 IC <sub>50</sub> (μM)
Met	No inhibition	No inhibition
Leu	No inhibition	45
Thr	0.5	0.3
Ser	2	0.05
Ala	10	0.01
Gly	15	0.03

Site-directed mutagenesis was used to change residue 109 in SAPK3 from methionine to one of five other amino acids. The results with SB 203580 are taken from [28].

The finding that ZM 336372 inhibits SAPK2/p38 (Table 1) as well as c-Raf and B-Raf was intriguing because SB 203580, a structurally unrelated compound that is a relatively specific inhibitor of SAPK2/p38, also inhibits c-Raf [27]. Moreover, like ZM 336372, SB 203580 also induces an activation of c-Raf in cells (measured in the absence of the drug) [27]. In this paper we demonstrate that the binding sites for ZM 336372 and SB 203580 on SAPK2/p38 (and presumably c-Raf and B-Raf) are likely to overlap because the single mutation of Thr106→Met abolishes inhibition by both compounds (Figure 8). A-Raf should also be sensitive to ZM 336372 because the critical threonine residue is also conserved in this isoform. In addition, other MAP kinase family members that are resistant to SB 203580 because they possess methionine instead of threonine at this position, can be made sensitive to either ZM 336372 or SB 203580 by changing this residue to threonine (Figure 8). Mutation to smaller residues (glycine and alanine), however, increases sensitivity to SB 203580, but decreases sensitivity to ZM 336372 (Table 4). We have excluded the possibility that SB 203580 triggers the activation of c-Raf by binding to SAPK2/p38 rather than c-Raf itself, because the concentration of SB 203580 required to activate c-Raf in cells is higher than that required to inhibit SAPK2/p38. In addition, SB 203580 still induces the activation of c-Raf in cells overexpressing an SB-203580-resistant mutant of SAPK2/p38 in which SB 203580 no longer prevents the activation of MAPKAP-K2 (a downstream target of SAPK2/p38) [27]. It could also be argued that the activation of c-Raf by ZM 336372 is mediated by the binding of this drug to SAPK2/p38 and not c-Raf itself. This is unlikely, however, because ZM 336372 has a much greater effect on the activation of c-Raf than SB 203580 does, an observation that correlates with its greater potency against the isolated c-Raf enzyme rather than its somewhat lower potency against SAPK2/p38.

Preincubation of cells with ZM 336372 does not trigger any activation of p42 MAPK (Figure 3), yet subsequent

exposure to EGF in the continued presence of ZM 336372, which only increases c-Raf activity by a further 20–30%, induces essentially complete activation of p42 MAPK (Figure 3) [22]. Similar results have been obtained with weaker activators of c-Raf in Swiss 3T3 cells, such as PDGF and phorbol esters [22] that, after preincubation with ZM 336372, trigger an almost equally strong activation of p42 MAPK, even though no further activation of c-Raf is detectable under these conditions. The simplest interpretation of these results is that c-Raf activity is not rate limiting for the activation of MKK1 and p42 MAPK by these agonists. It remains possible, however, that in addition to activating c-Raf *per se*, EGF and other agonists facilitate the activation of MKK1 in additional ways, for example, by inducing the inhibition of a MKK1 phosphatase or by activating kinase suppressor of Raf (KSR), another protein kinase that appears to be required for activation of the MAP kinase cascade but whose mechanism of action is unknown [32]. Whatever the explanation, however, these observations together with the failure of ZM 336372 to reverse the transformed phenotype of Ras or Raf-transformed cell lines (Table 2) suggests that compounds which inhibit the kinase activity of Raf might not be useful as anticancer drugs.

### Significance

Nearly all aspects of cell life are controlled by the reversible phosphorylation of proteins and abnormal protein phosphorylation underlies a number of major diseases. There is therefore increasing interest in the development of specific inhibitors of protein kinases with therapeutic potential. Inappropriate activation of the Ras/MAP kinase cascade is a cause of cancer and, in the present study, we developed a potent and relatively selective inhibitor of Raf, the protein kinase that lies at the head of this pathway. However, surprisingly, this inhibitor (ZM 336372) did not prevent activation of the Ras/MAP kinase cascade, nor did it reverse the phenotype of Ras-transformed cell lines. Further investigation revealed that inhibition by ZM 336372 in cells was also accompanied by a remarkable activation of Raf. Thus Raf appears to suppress its own activation by a novel feedback loop, such that inhibition is always counterbalanced by reactivation. These observations imply that some agonists reported to trigger the cellular activation of Raf might actually be inhibitors of this enzyme and that compounds which inhibit the kinase activity of Raf may not be useful as anticancer drugs. We also show that, although ZM 336372 did not inhibit 17 other protein kinases tested, it did inhibit stress-activated protein kinase 2a (SAPK2a) and SAPK2b (also called p38 and p38β), albeit less strongly. SAPK2a and SAPK2b are known to be inhibited relatively specifically by the anti-inflammatory drug SB 203580, and we have shown previously that SB 203580 inhibits Raf, although not as potently as SAPK2a/p38 and SAPK2b/p38β.



Interestingly, a point mutation in SAPK2a/p38 that renders it insensitive to SB 203580 also made it insensitive to ZM 336372, and the reverse mutation that makes other SAPK family members sensitive to SB 203580 also made them sensitive to ZM 336372. This suggests that the binding sites for SB 203580 and ZM 336372 are likely to overlap, despite their dissimilarity in structure.

## Materials and methods

### Materials

Ro 318220 was a kind gift from Dr D. Bradshaw (Roche Pharmaceutical Company, Welwyn Garden City, UK), PD 98059 was purchased from Calbiochem and wortmannin and PMA from Sigma. These drugs were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 10–50 mM. They were either diluted appropriately in aqueous buffers just prior to use, or added directly to the cell culture media to achieve final concentrations of 100 nM to 50  $\mu$ M. Control experiments contained the equivalent amounts of DMSO, which did not exceed 0.2% (v/v) in any experiment. This concentration did not affect the activity or activation of any protein kinase examined. EGF was purchased from Boehringer Mannheim and the monoclonal anti-H-Ras antibody (Y13 259) from Oncogene Science Products. Antibodies against c-Raf [24], MAPKAP-K1 [33] MKK1 and p42 MAPK were raised in sheep at the Scottish Antibody Production Unit (Carlisle, Lanarkshire, UK). The anti-MKK1 antibody was raised against a peptide corresponding to residues 2–17 (PKKKPTPIQLNPAPDG) and the anti-p42 MAPK antibody against the carboxy-terminal peptide (EETARFQPGYRS). All peptides were conjugated to both bovine serum albumin and keyhole limpet haemocyanin before injection and the antisera were affinity purified on appropriate immobilised peptide affinity columns.

### Western blotting

HCT 116 human colon carcinoma cells were obtained from ATCC (Manassas, Virginia, U.S.A.) MAPK antibody (Santa Cruz SC-94) was used according to the manufacturer's instructions.

### Cell culture and stimulation

Confluent mouse Swiss 3T3 fibroblasts, human 293 cells and monkey COS-1 cells were incubated for 18 h in DMEM containing 0.5% FCS. After treatment with growth factors or inhibitors as indicated, each 10 cm dish of cells was lysed in 0.4 ml of ice-cold Buffer A (20 mM Tris acetate, pH 7.5, 0.27 M sucrose, 1% (by mass) Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 10 mM sodium  $\beta$ -glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 0.1% (by vol) 2-mercaptoethanol, 0.1 mM phenylmethylsulphonyl fluoride, 1 mM benzamide, 5  $\mu$ g/ml leupeptin), and the lysates frozen immediately in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use. H-ras and c-raf transformed NIH3T3 cells were cultured in DMEM containing 5% charcoal stripped FCS. After 5 days, morphology was scored by microscopic observation and growth monitored by trypsinisation and cell counting.

### Immunoprecipitation of protein kinases

c-Raf was immunoprecipitated from 400  $\mu$ g cell lysate protein, MKK1 from 100  $\mu$ g of cell lysate and p42 MAPK and MAPKAP kinase-1 from 50  $\mu$ g cell lysate protein using 5  $\mu$ l of protein G-Sepharose conjugated to 4  $\mu$ g of the appropriate antibody [24,33].

### Assay of protein kinases

c-Raf, MKK1 and p42 MAPK [24] and MAPKAP kinase-1 [33] were assayed as described previously. c-Raf activity was measured in a coupled assay containing MKK1, p42 MAPK and its substrate myelin basic protein (MBP). One unit of c-Raf activity was that amount which increased the activity of p42 MAPK by 1 unit/min. MKK1 was assayed by the activation of bacterially expressed p42 MAPK [24]. One unit of MKK1 activity (U) was that amount which increased the activity of p42 MAPK by 1 unit/min. One unit of MAPK activity was that amount which catalysed the phosphorylation of 1 nmol of MBP in 1 min.

For the initial compound screen, assays were performed in microtitre plates in 50  $\mu$ l buffer as described previously [24] and modified to a single step reaction containing 0.3U Raf, 0.5  $\mu$ g GST-MKK1, 1  $\mu$ g GST-p42 MAPK, 16  $\mu$ g MBP and 0.3mCi- $[\gamma^{33}\text{P}]$  ATP (30  $\mu$ M). Assays were performed at room temperature for 90 min before being stopped by addition of 75  $\mu$ l 20% (v/v) phosphoric acid and captured on P30 filtermats (Wallac, Milton Keynes, UK) using a microcell harvester. Filters were washed and counted as described previously.

### Measurement of guanine nucleotides bound to Ras

Confluent Swiss 3T3 cells were incubated for 18 h in DMEM containing 0.5% FCS, then for 1 h in phosphate-free DMEM before labelling for 4 h with  $^{32}\text{P}$ -orthophosphate (5 mCi per 10 cm dish). After stimulation, cells were washed with ice-cold 20 mM Tris/HCl pH 7.5, 150 mM NaCl and lysed in Buffer B (50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM  $\text{MgCl}_2$ , 1 mg/ml bovine serum albumin (BSA), 1 mM sodium phosphate, complete proteinase inhibitor cocktail (Boehringer Mannheim), 100  $\mu$ M GTP, 100  $\mu$ M GDP, 1 mM ATP) containing 1% (by mass) Triton X-114 (Boehringer Mannheim). Nuclei were removed by centrifugation for 5 min at  $13,000\times g$ , and the supernatant incubated for 3 min at  $37^{\circ}\text{C}$  and centrifuged for 2 min at  $13,000\times g$  to separate the Triton X-114 and aqueous phases. The detergent phase was diluted tenfold with Buffer B plus 0.5 M NaCl. The lysate was pre-cleared with protein G-Sepharose coupled to rabbit pre-immune serum (15 min) and Ras immunoprecipitated using 25  $\mu$ l protein G-Sepharose beads coupled to 10  $\mu$ g anti-H-Ras antibody (60 min). The immunoprecipitates were washed 8 $\times$  with Buffer C (50 mM HEPES, pH 7.4, 500 mM NaCl, 5 mM  $\text{MgCl}_2$ , 0.1% Triton X-100, 0.005% SDS). GTP and GDP were eluted by incubation for 2 min at  $70^{\circ}\text{C}$  in 2 mM EDTA, 2 mM DTT, 0.2% (by mass) SDS, 0.5 mM GDP, 0.5 mM GTP and separated on polyethyleneimine (PEI) cellulose plates (Merck) developed in 1.2 M ammonium formate, 0.8 M HCl. Plates were autoradiographed and the radioactive spots cut out and analysed by Cerenkov counting [34].

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## References

- Marshall, C.J. (1995). Specificity of receptor tyrosine kinase signaling – transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**, 179–185.
- Ullrich, A. & Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase-activity. *Cell* **61**, 203–212.
- Lowenstein, E.J., *et al.*, & Schlessinger, J. (1992). The SH2 and SH3 domain containing protein Grb2 links receptor tyrosine kinases to Ras signaling. *Cell* **70**, 431–442.
- Pellicci, G., *et al.*, & Pellicci, P.G. (1992). A novel transforming protein (Shc) with an SH2 domain is implicated in mitogenic signal transduction. *Cell* **70**, 93–104.
- Rozakis-Adcock, M., *et al.*, & Pawson, T. (1992). Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature* **360**, 689–692.
- Li, N., *et al.*, & Schlessinger, J. (1993). Guanine-nucleotide-releasing factor mso1 binds to Grb2 and links receptor tyrosine kinases to Ras signaling. *Nature* **363**, 85–88.
- Gale, N.W., Kaplan, S., Lowenstein, E.J., Schlessinger, J. & Bar-Sagi, D. (1993). Grb2 mediates the EGF-dependent activation of guanine-nucleotide exchange on Ras. *Nature* **363**, 88–92.
- Bowtell, D., Fu, P., Simon, M. & Senior, P. (1992). Identification of murine homologs of the drosophila son of sevenless gene – potential activators of Ras. *Proc. Natl Acad. Sci. USA* **89**, 6511–6515.
- Clarke, S. (1992). Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. *Ann. Rev. Biochem.* **61**, 355–386.
- Traverse, S., Cohen, P., Paterson, H., Marshall, C., Rapp, U. & Grand, J.A. (1993). Specific association of activated MAP kinase kinase (Raf) with the plasma-membranes of Ras-transformed retinal cells. *Oncogene* **8**, 3175–3181.

11. Leever, S.J., Patterson, H.F. & Marshall, C.J. (1994). Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma-membrane. *Nature* **369**, 411-414.
12. Stokoe, D., Macdonald, S.G., Cadwallader, K., Symons, M. & Hancock, J.F. (1994). Activation of Raf as a result of recruitment to the plasma-membrane. *Science* **264**, 1463-1467.
13. Morrison, D.K. & Cutler, R.E. (1997). The complexity of Raf-1 regulation. *Curr. Opin. Cell Biol.* **9**, 174-179.
14. Chen, R.H., Sarnecki, C. & Blenis, J. (1992). Nuclear localisation and regulation of Erk encoded and Rsk encoded protein kinases. *Mol. Cell. Biol.* **12**, 915-927.
15. Traverse, S., Gomez, N., Paterson, H., Marshall, C. & Cohen, P. (1992). Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells – comparison of the effects of nerve growth-factor and epidermal growth-factor. *Biochem. J.* **288**, 351-355.
16. Lenormand, P., Sardet, C., Pages, G., L'Allemand, G., Brunet, A. & Pouyssegur, J. (1993). Growth-factors induce nuclear translocation of MAP kinases (p42 MAPK) and (p44 MAPK) but not of their activator MAP kinase kinase (p45 MAPKK) in fibroblasts. *J. Cell Biol.* **122**, 1079-1088.
17. Traverse, S., Seedorf, K., Paterson, H., Marshall, C.J., Cohen, P. & Ullrich, A. (1994). EGF triggers neuronal differentiation of PC12 cells that overexpress the EGF receptor. *Curr. Biol.* **4**, 694-701.
18. Law, N. & Lydon, N.B. (1996). *Emerging Drugs; The Prospect for Improved Medicines*. pp 241-261. Ashley Publications Ltd.
19. Bos, J.L. (1989). Ras oncogenes in human cancers - a review. *Cancer Res.* **49**, 4682-4689.
20. Rapp, U.R., et al., & Anderson, W.B. (1988). Raf family serine/threonine protein kinases in mitogen signal transduction. *Cold Spring Harbour Symp. Quant. Biol.* **53**, 173-184.
21. Sivaraman, V.S., Wang, H-Y., Nuovo, G.J. & Malbon, C.C. (1997). Hyperexpression of mitogen-activated protein kinase in human breast cancer. *J. Clin. Invest.* **99**, 1478-1483.
22. Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T. & Saltiel, A.R. (1995). PD 098059 is a specific inhibitor of the activation of MAP kinase kinase-1 *in vitro* and *in vivo*. *J. Biol. Chem.* **270**, 27489-27494.
23. Dudley, D.T., Pang, L., Decker, S.J., Bridges, A.J. & Saltiel, A.R. (1995). A synthetic inhibitor of the mitogen-activated protein-kinase cascade. *Proc. Natl Acad. Sci. USA* **92**, 7686-7689.
24. Alessi, D.R., Cohen, P., Ashworth, A., Cowley, S., Leever, S.J. & Marshall, C.J. (1995). Assay and expression of mitogen-activated protein kinase, MAP kinase kinase, and Raf. *Methods Enzymol.* **255**, 279-290.
25. Cross, D.A.E., Alessi, D.R., Vandenhede, J.R., McDowell, H.E., Hundal, H.S. & Cohen, P. (1994). The inhibition of glycogen-synthase kinase-3 by insulin or insulin-like growth factor-1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin - evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. *Biochem. J.* **303**, 21-26.
26. Alessi, D.R. (1997). The protein kinase C inhibitors Ro318220 and GF109203X are equally potent inhibitors of MAPKAP kinase-1b (Rsk-2) and p70 S6 kinase. *FEBS Lett.* **402**, 121-123.
27. Hall-Jackson, C.A., Goedert, M., Hedge, P. & Cohen, P. (1999). Effect of SB 203580 on the activity of c-Raf *in vitro* and *in vivo*. *Oncogene* **18**, 2047-2054.
28. Evers, P.A., Craxton, M., Morrice, N., Cohen, P. & Goedert, M. (1998). Conversion of SB 203580-insensitive MAP kinase family members to drug sensitive forms by a single amino-acid substitution. *Chem. Biol.* **5**, 321-328.
29. Wilson, K.P., et al., & Su, M.S.S. (1997). The structural basis for the specificity of pyridinylimidazole inhibitors of p38 MAP kinase. *Chem. Biol.* **4**, 423-431.
30. Gum, R.J., et al., & Young, P.R. (1998). Acquisition of sensitivity of stress-activated protein kinases to the p38 inhibitor, SB 203580, by alteration of one or more amino acids within the ATP binding pocket. *J. Biol. Chem.* **273**, 15605-15610.
31. Fox, T., et al., & Wilson, K.P. (1998). A single amino acid substitution makes ERK2 susceptible to pyridinyl imidazole inhibitors of p38 MAP kinase. *Protein Sci.* **7**, 2249-2255.
32. Downward, J. (1995). KSR: A novel player in the RAS pathway. *Cell* **83**, 831-834.
33. Tan, Y., Rouse, J., Zhang, A., Cariati, S., Cohen, P. & Comb, M. (1996). FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. *EMBO J.* **15**, 4629-4642.
34. Burgering, B.M.T., et al., & Bos, J.L. (1991). Insulin stimulation of gene-expression mediated by p21 Ras activation. *EMBO J.* **10**, 1103-1109.
35. Hedge P.J. & Boyle F.T. (1998). Use of phenylamido derivatives as raf kinase inhibitors - for treatment of tumours. Issue date 18/06/98. Int. patent application WO 98/22103.

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