Specificity and mechanism of action of some commonly used protein kinase inhibitors

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The specificities of 28 commercially available compounds reported to be relatively selective inhibitors of particular serine/threonine-specific protein kinases have been examined against a large panel of protein kinases. The compounds KT 5720, Rottlerin and quercetin were found to inhibit many protein kinases, sometimes much more potently than their presumed targets, and conclusions drawn from their use in cell-based experiments are likely to be erroneous. Ro 318220 and related bisindoylmaleimides, as well as H89, HA1077 and Y 27632, were more selective inhibitors, but still inhibited two or more protein kinases with similar potency. LY 294002 was found to inhibit casein kinase-2 with similar potency to phosphoinositide (phosphatidylinositol) 3-kinase. The compounds with the most impressive selectivity profiles were KN62, PD 98059, U0126, PD 184352, rapamycin, wortmannin, SB 203580 and SB 202190. U0126 and PD 184352, like PD 98059, were found to block the mitogen-activated protein kinase (MAPK) cascade in cell-based assays by preventing the activation of MAPK kinase (MKK1), and not by inhibiting MKK1 activity directly. Apart from rapamycin and PD 184352, even the most selective inhibitors affected at least one additional protein kinase. Our results demonstrate that the specificities of protein kinase inhibitors cannot be assessed simply by studying their effect on kinases that are closely related in primary structure. We propose guidelines for the use of protein kinase inhibitors in cell-based assays.

Key words: PD 98059, PD 184352, protein phosphorylation, SB 203580, U0126.

INTRODUCTION

Nearly all aspects of cell life are controlled by the reversible phosphorylation of proteins. About one-third of mammalian proteins contain covalently bound phosphate, and there are likely to be 1000 protein kinases encoded by the human genome. If there are 100000 gene products in total, then an 'average' protein kinase phosphorylates about 30 proteins. This number is, however, likely to be an underestimate, because many proteins are phosphorylated by two or more protein kinases. A major challenge in this field is therefore to identify the physiological substrates of each protein kinase.

In recent years, several small, cell-permeant inhibitors of protein kinases have been developed that exhibit a relatively high degree of specificity for a particular protein kinase, and which may be useful for identifying the physiological substrates and cellular functions of these enzymes (reviewed by Cohen [1]). They include rapamycin, an inhibitor of the protein kinase mTOR (mammalian target of rapamycin, also called FRAP) [2], an immunosuppressant that has recently been approved for clinical use to prevent tissue rejection after kidney transplantation. They also include inhibitors of receptor tyrosine kinases, such as SU 101 [3] and CGP 57148 [4], which are relatively specific inhibitors of platelet-derived growth factor receptor function or activity, and ZD 1839 [5] and CP 358774 [6], which inhibit the epidermal growth factor (EGF) receptor. These compounds are currently undergoing human clinical trials for the treatment of cancers in which the receptors for platelet-derived growth factor and EGF are overexpressed. SU 5416 [7] and PD 173074 [8], which are inhibitors of the vascular endothelial growth factor and fibroblast growth factor receptor tyrosine kinases respectively, suppress angiogenesis and thereby destroy the vasculature that is critical for the growth and proliferation of tumour cells. SU 5416 has entered phase III clinical trials for the treatment of several types of cancer.

Two further compounds, SB 203580 and PD 98059, are inhibitors of mitogen-activated protein kinase (MAPK) cascades and have been extremely useful for identifying some of the physiological roles of the cell signalling pathways that they inhibit [9]. The pyridinyl imidazole SB 203580 inhibits the MAPK

Abbreviations used: AMPK, AMP-activated protein kinase; CAM-K, calmodulin-dependent protein kinase; CHK, checkpoint kinase; CK2, casein kinase 2; CREB, cAMP-response element binding protein; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; GSK3, glycogen synthase kinase 3; GST, glutathione S-transferase; HMG, high-mobility group; JNK, c-Jun N-terminal kinase; LCK, lymphocyte kinase; MAPK, mitogen-activated protein kinase; MAPKAP-K1, MAPK-activated protein kinase-1; MAPKAP-K2, MAPK-activated protein kinase 2; MKK, MAPK kinase (also called MEK); MLCK, myosin light chain kinase; MNK, MAPK-integrating kinase; MSK1, mitogen- and stress-activated protein kinase 1; mTOR, mammalian target of rapamycin; PDK1, 3-phosphoinositide-dependent protein kinase 1; PI 3-kinase, phosphoinositide (phosphatidylinositol) 3-kinase; PKA, cAMP-dependent protein kinase; PKB, protein kinase B (also called Akt); PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PHK, phosphorylase kinase; PRAK, p38-regulated/activated kinase; PRK, protein kinase C-related protein kinase; ROCK, Rho-dependent protein kinase; SAPK2a, stress-activated protein kinase 2a (also called p38); SAPK2b, stress-activated protein kinase 2b (also called p38β2); SAPK3, stress-activated protein kinase 3 (also called p38γ); SAPK4, stress-activated protein kinase 4 (also called p38δ); SGK, serum- and glucocorticoid-induced kinase; S6K1, p70 ribosomal protein S6 kinase; Sk, skeletal muscle; Sm, smooth muscle.

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family member termed stress-activated protein kinase 2a (SAPK2a; also known as p38) [10], which lies in a pathway that is activated strongly by cellular stresses, pro-inflammatory cytokines and bacterial lipopolysaccharide. The high degree of specificity of SB 203580 is indicated by its failure to affect the activities of a number of other protein kinases [10], including closely related MAPK family members, and the molecular basis for this selectivity has been elucidated [11,12]. Pyridinyl imidazoles that are closely related to SB 203580 show efficacy in animal models of chronic inflammatory diseases, such as rheumatoid arthritis [13]. PD 98059 prevents the activation of MAPK kinase 1 (MKK1; also known as MEK1) [14], a component of the classical MAPK cascade, which is stimulated most strongly by growth factors and tumour-promoting phorbol esters. PD 98059 reverses the transformed phenotype of Ras-transformed cell lines [15], while PD 184352, reported to be a much more potent inhibitor of MKK1, inhibits by 80% the growth of human colon tumours implanted into mice [16]. PD 98059 binds to MKK1, preventing its activation by 'upstream' protein kinases, such as Raf [14]. PD 98059 is therefore not a protein kinase inhibitor, but a compound that stops one protein kinase (Raf) from activating another (MKK1).

The availability of more cell-permeant protein kinase inhibitors would be extremely useful in helping to delineate the physiological roles of these enzymes. A number of other compounds have been reported to inhibit particular serine/threonine protein kinases specifically, and are being used extensively in cell-based assays to invoke physiological roles for the protein kinases that they are presumed to inhibit. However, the specificity of many of these compounds has not been tested rigorously. In this paper we have used a large panel of protein kinases to examine the specificities of many compounds reported to be relatively specific inhibitors of particular serine/threonine protein kinases. This analysis has led to some surprising results.

MATERIALS AND METHODS

Materials other than protein kinases

PD 98059, SB 203580, SB 202190, Ro 318220, U0126, Rottlerin, KN62, H89, K252c, KT 5720, UCN1, rapamycin, quercetin, chelerythrine, LY 294002, Go6976 and 10-[3-(1-piperazinyl)-propyl]-2-trifluoromethyl-phenothiazine were purchased from Calbiochem–Novabiochem Corp. (La Jolla, CA, U.S.A.); phosphatidylserine was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, U.S.A.); EGF, tissue culture reagents, Sf21 cells, myelin basic protein and histone H1 were from Life Technologies (Paisley, Scotland, U.K.); wortmannin, 1,2-dioleoyl-*sn*-glycerol and the peptide LRRASLG (Kemptide) were from Sigma (Poole, Dorset, U.K.); HA1077 was from Research Biochemicals International (Natick, MA, U.S.A.); and the bisindolylmaleimides Bis-1, Bis-3, Bis-4, Bis-5, Bis-8 and Bis-10 were from Alexis Corp. (U.K.) Ltd (Nottingham, U.K.). Protein G–Sepharose was from Amersham Pharmacia Biotech Inc. (Little Chalfont, Bucks., U.K.). The peptide KKRARRATSNVFA was obtained from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, U.S.A.), and all other peptides were synthesized by Dr Graham Bloomberg (Department of Biochemistry, University of Bristol, Bristol, U.K.) or by Mr F. B. Caudwell in this Unit. Y 27632 was purchased from QuChem Ltd (The Queen's University, Belfast, U.K.). Glycogen phosphorylase *b* was isolated from rabbit skeletal muscle by Dr N. Morrice in this Unit, and calmodulin was isolated from bovine brain. Skeletal muscle heavy meromyosin was generously donated by Dr M. Ikebe (Case Western Reserve University, Cleveland, OH, U.S.A.).

Source and purification of kinases

Unless stated otherwise, all protein kinases were of human origin and were expressed either as glutathione S-transferase (GST) fusion proteins in *Escherichia coli* (purified by Dr C. Armstrong, Dr J. Leitch and Miss F. Douglas in this Division) or as hexahistidine (His_6)-tagged proteins in insect Sf9 or Sf21 cells (purified by Dr C.Armstrong, Dr. A. Paterson and Miss G. Wiggin in this Division). GST fusion proteins were purified by affinity chromatography on glutathione–Sepharose, and His_6-tagged proteins were purified on nickel/nitrilotriacetate–agarose.

Expressed in *E. coli*

The following kinases were expressed in *E. coli*: MKK3, MKK4, MKK6, MKK7β, rabbit MKK1, murine extracellular-signal-regulated kinase 2 (ERK2) and human c-Jun N-terminal kinase (JNK) 1α1, SAPK2a/p38, SAPK2b/p38β2, SAPK3/p38γ, SAPK4/p38δ, MAPK-activated protein kinase 2 (MAPKAP-K2), checkpoint kinase 1 (CHK1) and CHK2. CHK2 contained an additional six histidine residues at its C-terminus to aid purification of the full-length protein.

Expressed in Sf9 cells

The following kinases were expressed in Sf9 cells: protein kinase Bα (PKBα; also known as Akt), [Ser-422 $\rightarrow$ Asp]SGK (serum- and glucocorticoid-induced kinase), p38-regulated/activated kinase (PRAK), rat ROCK-II-(1–543) (where ROCK is Rho-dependent protein kinase), mitogen- and stress-activated protein kinase 1 (MSK1), casein kinase 2 (CK2) (holoenzyme produced by co-expression of the α and β subunits), lymphocyte kinase (LCK) and the p110 catalytic subunit of bovine phosphoinositide (phosphatidylinositol) 3-kinase (PI 3-kinase) γ.

Expressed in Sf21 cells

The following kinases were expressed in Sf21 cells: 3-phosphoinositide-dependent protein kinase 1 (PDK1), glycogen synthase kinase 3β (GSK3β), [Thr-412 $\rightarrow$ Glu]S6K1 (p70 ribosomal protein S6 kinase) lacking the C-terminal 104 residues, and a constitutively active mutant of the catalytic domain of c-Raf in which tyrosines 340 and 341 were both mutated to aspartic acid. The Raf construct was a GST fusion protein fused to an N-terminally truncated version of c-Raf starting at Gln-307.

Expressed in human embryonic kidney 293 cells

GST–PRK2-(501–984) (where PRK is protein kinase C-related protein kinase) was expressed in 293 cells and purified on glutathione–Sepharose.

Tissue purified

Phosphorylase kinase (PHK) (Dr C. MacKintosh), MAPKAP-K1b (also known as RSK2) and skeletal muscle myosin light chain kinase (SkMLCK) (Dr N. Morrice) were purified from rabbit skeletal muscle, cAMP-dependent protein kinase (PKA) was purified from bovine heart (Dr. C. Smythe) and AMP-activated protein kinase (AMPK) was purified from rat liver (Dr S. Davies) in this Division by the investigators indicated in parentheses. MLCK from chicken gizzard smooth muscle (SmMLCK) was generously donated by Dr M. Ikebe (Case Western Reserve University, Cleveland, OH, U.S.A.).

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Purchased

Protein kinase Cα (PKCα) and PKCδ were purchased from PanVera Corp. (Madison, WI, U.S.A.), and CAM-KII was from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, U.S.A.).

Activation of protein kinases

JNK1α1 was activated with MKK4; SAPK2a/p38, SAPK2b/p38β2, SAPK3/p38γ and SAPK4/p38δ with MKK6; PKBα, SGK and S6K1 with PDK1; ERK2 with MKK1; MAPKAP-K1b/RSK2, MAPKAP-K2, PRAK and MSK1 with ERK2; MKK1 with c-Raf; and c-Raf by co-transfection with LCK and oncogenic Ras in Sf9 cells.

Kinase assays

All protein kinase activities were linear with respect to time in every incubation. Assays were performed either manually for 10 min at 30 °C in 50 μl incubations using [γ-^{32}P]ATP, or with a Biomek 2000 Laboratory Automation Workstation in a 96-well format (Beckman Instruments, Palo Alto, CA, U.S.A.) for 40 min at ambient temperature in 25 μl incubations using [γ-^{33}P]ATP. The concentrations of ATP and magnesium acetate were 0.1 mM and 10 mM respectively, unless stated otherwise. This concentration of ATP is 5–10-fold higher than the K_m for ATP of most of the protein kinases studied in the present paper, but lower than the normal intracellular concentration, which is in the millimolar range. All assays were initiated with MgATP. Manual assays were terminated by spotting aliquots of each incubation on to phosphocellulose paper, followed by immersion in 50 mM phosphoric acid. Robotic assays were terminated by the addition of 5 μl of 0.5 M phosphoric acid before spotting aliquots on to P30 filter mats (Wallac). All papers were then washed four times in 50 mM phosphoric acid to remove ATP, once in acetone (manual incubations) or methanol (robotic incubations), and then dried and counted for radioactivity.

GSK3β, S6K1, MAPKAP-K1b/RSK2, PKA, CHK1, CHK2, MSK1 and SGK were assayed in 8 mM Mops, pH 7.0, containing 0.2 mM EDTA. Substrate peptides (single-letter code for amino acids) were: GSK3, YRRAAVPPSPSLSRHSSPHQS(PO_4)-EDEEE (20 μM); S6K1, KKRNRTLTV (100 μM); MAPKAP-K1b/RSK2, KKKNRTLSVA (30 μM); PKA, LRRASLG (30 μM); CHK1 and CHK2, KKKVSRSGLYRSPSMPENL-NRPR (200 μM); MSK1 and SGK, GRPRTSSFAEG (30 μM). PKBα was also assayed with GRPRTSSFAEG (30 μM) in 50 mM Tris/HCl, pH 7.5, containing 0.05 % 2-mercaptoethanol. ERK2, SAPK2a/p38, SAPK2b/p38β2, SAPK3/p38γ and SAPK4/p38δ were assayed in 25 mM Tris/HCl, pH 7.5, containing 0.1 mM EGTA, with myelin basic protein (0.33 mg/ml) as substrate. MAPKAP-K2 and PRAK were assayed in 50 mM sodium β-glycerophosphate, pH 7.5, containing 0.1 mM EGTA, with the peptides KKLNRTLSVA (30 μM) and KKLRRTLSVA (30 μM) respectively as substrate. CAM-KII was assayed in 50 mM Hepes, pH 7.4, containing 5 mM $CaCl_2$ and 0.03 mg/ml calmodulin, with the peptide KKLNRTLSVA (60 μM) as substrate. JNK1, ROCK-II and PRK2 were assayed in 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA and 0.1 % 2-mercaptoethanol with the following peptides: JNK1, 3 μM GST–ATF2-(19–96) (where ATF2 is activating transcription factor 2); PRK2, 30 μM AKRRRLSSLRA; ROCK-II, KEAKEKRQEQIAKRRRLSS-LRASTSKSGGSQK. MLCK was assayed as for JNK1, ROCK-II and PRK2, except that EGTA was replaced by 0.1 mM $CaCl_2$, calmodulin (0.1 μM) was included and the substrate was skeletal muscle heavy meromyosin (0.5 mg/ml) (SkMLCK) or 100 μM KKRAARATSNVFA (SmMLCK). AMPK was assayed in 50 mM Hepes, pH 7.4, 1 mM dithiothreitol, 0.02 % Brij-35 and 0.2 mM AMP, with HMRSAMSGLHLVKRR (0.2 mM) as substrate. PKCα was assayed in 20 mM Hepes, pH 7.4, 0.03 % Triton X-100, 0.1 mM $CaCl_2$, 0.1 mg/ml phosphatidylserine and 10 μg/ml 1,2-dioleoyl-*sn*-glycerol, with 0.1 mg/ml histone H1 as substrate. PKCδ was assayed in an identical manner to PKCα, except that $CaCl_2$ was replaced with 0.1 mM EGTA. CK2 was assayed in 20 mM Hepes, pH 7.6, 150 mM NaCl, 0.1 mM EDTA, 5 mM dithiothreitol and 0.1 % Triton X-100, with RRRDDD-SDDD (165 μM) as substrate. LCK was assayed in 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA and 0.1 mM Na_3VO_4, using KVEKIGEGTYGVVYK (250 μM) as substrate. PHK was assayed in 50 mM Tris, 50 mM sodium β-glycerophosphate, pH 8.6, and 0.04 mM $CaCl_2$, using phosphorylase b (0.5 mg/ml) as substrate. MKK1 was assayed via its ability to activate ERK2 (0.07 mg/ml) in incubations containing 25 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.1 % 2-mercaptoethanol, 0.01 % Brij-35 and MgATP. After incubation for 15 min at 30 °C, activated ERK2 was assayed as described above. PDK1 was assayed in 50 mM Tris/HCl, pH 7.5, containing 0.1 % 2-mercaptoethanol, using the peptide KTFCGTPEYLAPEVRREPRILSEEEQEM-FRDFDYIADWC (PDKtide) as substrate. PI 3-kinase was assayed as described previously [17], except that the ATP concentration was increased to 0.1 mM and the lipid vesicles comprised 225 μM phosphatidylserine and 75 μM phosphatidylinositol.

RESULTS

H89

H89 is marketed by Calbiochem as ‘a selective and potent inhibitor of PKA’. In our standard assay conducted at 0.1 mM ATP, H89 (at 10 μM) inhibited eight protein kinases in our panel by 80–100 % (Table 1). IC_{50} values were determined for the protein kinases that were inhibited most strongly, and these studies revealed that three (MSK1, S6K1 and ROCK-II) were inhibited with a potency similar to or greater than that for PKA (Table 2).

Y 27632

This compound inhibits smooth muscle contractility and shows efficacy in normalizing high blood pressure in several rat models of hypertension [18]. It was originally reported to inhibit two isoforms of a Rho-dependent protein kinase, termed ROCK-I and ROCK-II [18], and to exert its anti-hypertensive effect by preventing ROCK from inhibiting smooth muscle protein phosphatase 1M [19], the major myosin phosphatase of this tissue. This leads to decreased phosphorylation of myosin, arterial smooth muscle relaxation and hence vasodilation of blood vessels. Y 27632 has also been shown to inhibit RhoA-mediated cell transformation [20], tumour cell invasion [21] and neutrophil chemotaxis [22]. These findings raised the possibility that inhibitors of ROCK may have additional/alternative therapeutic uses as anti-cancer or anti-inflammatory agents.

More recently, a very closely related molecule, Y 32885, was found to inhibit PRK1, a distinct Rho-dependent protein kinase, at a concentration similar to that which inhibits ROCK isoforms [23]. In the present study, we found that Y 27632 inhibited PRK2 (a kinase that is closely related to PRK1) with a potency similar to that for ROCK-II (Tables 1 and 2). The catalytic domains of PRK1/PRK2 only share 35–38 % amino acid sequence identity with ROCK-I/ROCK-II. Three other protein kinases were inhibited by Y 27632, but with IC_{50}

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Table 1 Inhibition of protein kinases by commercially available inhibitors

The inhibitor concentrations used are shown in parentheses. Results are presented as kinase activity as a percentage of that in control incubations (averages of duplicate determinations). ATP was present at 0.1 mM in all assays.

	Activity (% of control)															
Protein kinase	H89 (10 μM)	Y 27632 (10 μM)	HA1077 (20 μM)	Rottlerin (20 μM)	KN62 (10 μM)	U0126 (10 μM)	PD 184352 (10 μM)	PD 98059 (50 μM)	SB 203580 (10 μM)	SB 202190 (10 μM)	Wortmannin (1 μM)	LY 294002 (50 μM)	Quercetin (20 μM)	Rapamycin (1 μM)	LiCl (10 mM)	KCl (10 mM)
(a) Core panel																
MKK1	90±4	103±0	89±3	106±2	101±1	56±1	5±1	89±1	99±1	93±1	96±1	101±1	94±3	99±1	116±1	109±1
MAPK2/ERK2	87±1	94±3	94±8	139±17	92±1	92±3	107±5	85±3	85±6	89±6	90±12	114±13	113±8	90±16	107±2	102±2
JNK1α1/SAPK1c	97±1	98±1	96±1	49±1	104±4	96±4	102±2	111±2	101±5	93±1	97±1	108±0	101±5	98±6	92±0	89±1
SAPK2a/p38	99±1	94±5	93±3	111±5	95±6	75±1	100±3	85±5	2±1	0±0	86±5	98±3	138±7	93±12	108±1	102±1
SAPK2b/p38β2	97±4	107±3	97±15	127±3	98±1	90±4	119±1	95±6	10±1	3±0	74±3	98±0	150±6	88±10	96±1	104±1
SAPK3/p38γ	106±2	100±8	87±2	146±4	95±1	100±2	100±1	96±2	96±2	80±2	75±5	97±2	132±1	100±1	99±1	108±2
SAPK4/p38δ	105±3	95±1	103±11	130±1	110±22	111±1	98±3	94±8	93±4	87±9	79±11	94±1	103±3	82±12	84±2	99±2
MAPKAP-K1b	16±1	72±13	37±6	79±1	89±18	88±3	86±3	93±9	83±10	95±8	92±3	70±2	20±3	95±17	95±2	78±1
MAPKAP-K2	99±12	99±3	90±1	5±2	59±5	102±2	98±6	95±6	93±2	97±1	102±5	74±5	90±3	125±7	72±4	98±1
MSK1	3±1	57±2	19±2	38±3	81±10	104±1	118±3	86±3	86±2	88±3	99±4	83±7	37±3	104±2	104±6	105±5
PRAK	81±4	104±11	91±19	6±5	36±1	93±3	71±4	108±6	112±4	88±11	85±4	68±6	51±2	74±10	76±3	104±3
PKA	2±1	91±2	35±2	17±3	94±9	95±2	105±1	106±4	96±4	66±9	97±5	91±4	104±6	104±0	96±1	96±1
PKCα	79±1	98±0	86±1	95±2	95±5	92±2	99±1	93±4	89±4	92±0	100±2	91±4	70±1	99±5	98±1	97±1
PDK1	104±3	115±12	92±2	36±2	70±7	99±1	85±2	86±4	89±4	87±9	88±9	76±4	81±4	110±5	105±1	98±7
PKBα	17±1	90±0	88±5	27±3	67±8	79±5	89±4	82±1	62±1	53±2	96±6	60±2	99±2	91±0	95±5	96±6
SGK	25±1	109±5	92±5	81±0	78±5	91±1	111±14	90±1	83±5	98±1	101±7	72±1	35±0	108±6	99±1	100±1
S6K1	0±0	94±3	32±1	98±7	93±5	92±3	86±0	100±2	87±1	75±1	106±3	81±7	25±0	109±2	95±2	101±2
GSK3β	107±2	92±9	90±5	13±1	38±4	105±3	83±3	101±1	66±3	61±6	85±10	53±1	30±1	89±9	58±3	99±2
ROCK-II	0±0	13±2	7±1	88±7	88±0	94±1	107±4	80±3	77±1	61±2	91±1	104±10	55±2	92±2	101±2	102±1
AMPK	19±1	95±0	77±1	98±1	97±0	85±4	89±3	97±4	96±2	94±0	106±1	103±0	16±0	106±0	106±1	105±1
CK2	104±2	98±3	102±3	103±6	103±0	107±1	96±1	87±3	97±1	93±1	98±3	18±1	19±3	104±7	73±5	112±1
PHK	51±3	81±9	58±1	63±3	106±3	101±1	117±15	87±4	104±13	91±1	100±4	44±10	32±4	103±4	96±2	93±5
LCK	76±8	109±0	94±0	70±3	92±1	87±1	99±5	85±8	32±3	37±0	95±4	85±8	83±11	102±2	99±4	105±2
CHK1	21±1	99±1	82±6	107±3	104±2	95±1	104±3	99±4	95±2	95±1	99±3	90±2	56±1	102±2	96±3	97±1
(b) Other kinases																
CAM-KII					0±0											
SkMLCK			93±1								104±1					
SmMLCK			93±1		96±3						4±1					
PKCδ				101±1												
MKK3	95±1					114±4									94±1	109±2
MKK4	80±3					81±3									87±2	94±5
MKK6	86±1					79±1									108±5	113±9
MKK7	91±2					89±1									100±1	102±3
PI 3-kinase											0±0	13±0	18±2			
PRK2		6±1	15±1													

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Table 2 Concentrations of compounds required for 50% inhibition of the various protein kinases

The ATP concentration was 0.1 mM in all assays.

Compound	Protein kinase	IC_{50}
H89	S6K1	80 nM
H89	MSK1	120 nM
H89	PKA	135 nM
H89	ROCK-II	270 nM
H89	PKBα	2.6 μM
H89	MAPKAP-K1b	2.8 μM
Y 27632	ROCK-II	800 nM
Y 27632	PRK2	600 nM
Y 27632	MSK1	8.3 μM
Y 27632	MAPKAP-K1b	19 μM
Y 27632	PHK	44 μM
HA1077	ROCK-II	1.9 μM
HA1077	PRK2	4 μM
HA1077	MSK1	5 μM
HA1077	MAPKAP-K1b	15 μM
Rottlerin	PRAK	1.9 μM
Rottlerin	MAPKAP-K2	5.4 μM
KN62	CAM-KII	500 nM
U0126	MKK1	13 μM
U0126	PRAK	50 μM
U0126	SAPK2a/p38	55 μM
U0126	PKBα	60 μM
PD 184352	MKK1	300 nM
SB 202190	SAPK2a/p38	50 nM
SB 202190	SAPK2b/p38β2	100 nM
Wortmannin	SmMLCK	260 nM
LY 294002	PI3K	10 μM
LY 294002	CK2	6.9 μM
Ro 318220	GSK3β	38 nM
Ro 318220	S6K1	15 nM
Ro 318220	MAPKAP-K1b	3 nM
Ro 318220	MSK1	8 nM
Ro 318220	PKCα	33 nM
KT 5720	PHK	11 nM
KT 5720	PDK1	300 nM
KT 5720	PKA	3.3 μM

values 10-fold (MSK1), 24-fold (MAPKAP-K1b/RSK2) and 50-fold (PHK) higher than those for PRK2 or ROCK-II (Tables 1 and 2). Y 27632 had a minimal effect on the other protein kinases in the panel (Table 1).

HA1077

This compound, also known as AT877 or fasudil hydrochloride, shows efficacy for the treatment of cerebral vasospasm, without obvious side effects [24,25]. It is reported to inhibit several protein kinases, including PRK1 [23] and ROCK [22]. As for Y 27632, the clinical efficacy of HA1077 may be related to its ability to inhibit ROCK and/or PRK isoforms.

ROCK-II was the protein kinase in our panel that was inhibited most potently by HA1077 (Table 1). PRK2 and MSK1 were inhibited 2–3-fold less potently, and MAPKAP-K1/RSK 8-fold less potently (Table 2). S6K1 and PKA were inhibited with a similar potency to MAPKAP-K1/RSK (Table 1). Other protein kinases in the panel were inhibited minimally or not at all.

Rottlerin

Rottlerin is reported to inhibit PKC isoforms, especially PKCδ and CAM-KIII [26]. It has been used in many studies to implicate PKCδ in a variety of cellular events. However, in our standard assays at 0.1 mM ATP (and even at 0.01 mM ATP), Rottlerin had virtually no effect on PKCα or PKCδ activity, even at 20 μM. This was the case whether PKCs were assayed in the presence of phosphatidylserine using either histone H1 or myelin basic protein as a substrate (Table 1), or in the absence of lipid using protamine as a substrate (results not shown). The activity of PKCα towards histone H1 was dependent on phosphatidylserine and calcium ions, while the activity of PKCδ was phospholipid-dependent but calcium-independent, as expected from the known regulatory properties of these enzymes. In contrast, Rottlerin inhibited a number of protein kinases in our panel (Table 1). PRAK and MAPKAP-K2 were inhibited the most potently, with IC_{50} values of 1.9 μM and 5.4 μM respectively (Table 2).

KN62

This compound is reported to inhibit CAM-KII by interacting with the calmodulin-binding site on the enzyme [27], and has been used in many studies to implicate this protein kinase in the regulation of a variety of cellular events. KN62 inhibited CAM-KII with an IC_{50} of 500 nM in our standard assay. GSK3β and PRAK were inhibited 10-fold less potently and MAPKAP-K2 approx. 30-fold less strongly than CAM-KII, but the other protein kinases in the panel were inhibited minimally or not at all. Two other calmodulin-dependent protein kinases, PHK and SmMLCK, were not inhibited by KN62. However, KN62 is reported to inhibit CAM-KI and CAM-KIV at similar concentrations to CAM-KII [27a].

U0126

This compound is reported to inhibit MKK1 [28] and is being used widely to suppress activation of the classical MAPK cascade in cells [29]. U0126 was found to inhibit MKK1 five times more potently than SAPK2a/p38, PRAK and PKBα, while the other protein kinases in our panel were not inhibited (Table 1).

In our assays, the IC_{50} for inhibition of MKK1 was 13 μM (Table 2), 40 times higher than the concentration required to inhibit the EGF-induced activation of ERK2 (a physiological substrate of MKK1) or MAPKAP-K1 (a physiological substrate of ERK2) in Swiss 3T3 cells (Figure 1A) or the lipopolysaccharide-induced activation of MAPKAP-K1 in RAW264 murine macrophages (results not shown). These observations led us to discover that U0126 exerts its effects on cells by suppressing the activation of MKK1, and not by blocking its activity (Figure 1A). The concentration of U0126 that suppressed the activation of MKK1 by 50% in Swiss 3T3 cells was 70 nM, almost 200-fold lower than that required to inhibit MKK1 activity by 50% (Table 2). We confirmed this result by demonstrating that U0126 inhibits the activation of MKK1 by c-Raf *in vitro*, at much lower concentrations than those that inhibit MKK1 activity *per se* (Figure 1B). This inhibition is caused by the binding of U0126 to MKK1, and not to c-Raf, because U0126 does not inhibit the phosphorylation of myelin basic protein by Raf (Figure 1B). However, U0126 does not suppress the Raf-induced activation of MKK1 completely, raising the possibility that, in cells, the binding of U0126 to MKK1 also increases the rate at which it is dephosphorylated.

PD 184352

This compound is reported to inhibit MKK1 and to strongly suppress the growth of human colon tumours implanted into mice [16]. PD 184352 at 10 μM did not inhibit any other protein kinase in our panel significantly (Table 1).

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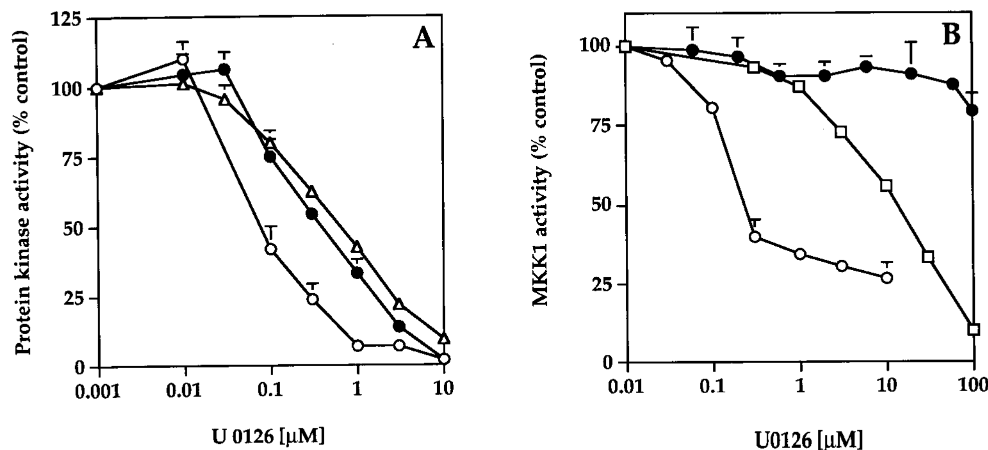

Figure 1 Effects of U0126 on the activation and activity of MKK1

(**A**) Effects of U0126 on the EGF-induced activation of MKK1, ERK2 and MAPKAP-K1/RSK in Swiss 3T3 cells. Murine Swiss 3T3 cells were cultured, stimulated and lysed as described previously [50]. The cells were incubated overnight in 0.5% foetal calf serum, incubated for 1 h in the absence or presence of the indicated concentrations of U0126 and stimulated with 100 ng/ml EGF for 3 min (MKK1), 5 min (ERK2) or 10 min (MAPKAP-K1/RSK). MKK1 (○), ERK2 (●) and MAPKAP-K1/RSK (△) were then assayed in the absence of U0126 after immunoprecipitation from 50 μg of lysate protein [14,53,54]. The results are presented relative to control incubations in which the inhibitor was omitted. Similar results were obtained in two further experiments. (**B**) Effects of U0126 on the activity of MKK1 and its activation by c-Raf *in vitro*. Purified MKK1 was incubated for 10 min with the indicated concentrations of U0126 and then assayed by its ability to activate ERK2 (□) as described in [14]. c-Raf was assayed by its ability to phosphorylate 0.3 μM MKK1 (○) and 0.33 mg/ml myelin basic protein (●) [14]. For MKK1, the reactions were terminated with SDS, subjected to SDS/PAGE and the ^{32}P-labelled MKK1 band excised and the radioactivity counted. The results are presented relative to control incubations in which the inhibitor was omitted. Similar results were obtained in three further experiments.

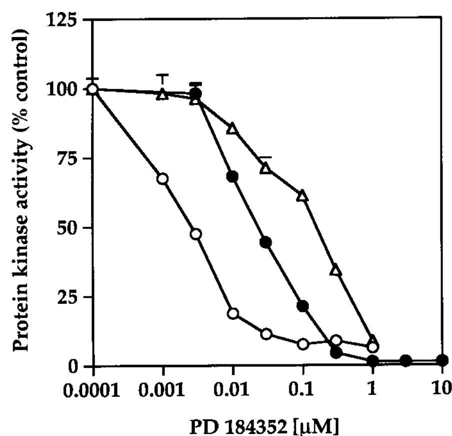

Figure 2 Effects of PD 184352 on the EGF-induced activation of MKK1, ERK2 and MAPKAP-K1/RSK in Swiss 3T3 cells

The experiment was carried out as in Figure 1(A), except that PD 184352 replaced U0126. MKK1 (○), ERK2 (●) and MAPKAP-K1/RSK (△) were then assayed in the absence of PD 184352 after immunoprecipitation from 50 μg of lysate protein.

The IC_{50} for inhibition of MKK1 by PD 184352 was 0.3 μM in our assays (Table 2), 15-fold higher than the concentration required to inhibit the EGF-induced activation of ERK2 in Swiss 3T3 cells (Figure 2). These observations led us to discover that, like U0126, PD 184352 exerts its effects on cells by suppressing the activation of MKK1, and not by blocking its activity (Figure 2). The activation of MKK1 in cells was inhibited by 50% at 2 nM PD 184352, i.e. a concentration over 100-fold lower than that which inhibits MKK1 *in vitro*. PD 184352 also inhibited the Raf-catalysed phosphorylation of MKK1 *in vitro* without any effect on the Raf-catalysed phosphorylation of myelin basic protein (results not shown).

PD 98059 and SB 203580

Information about the specificities of these well characterized protein kinase inhibitors was extended. No protein kinase was inhibited by PD 98059 at a concentration (50 μM) that prevents activation of the classical MAPK cascade in cell-based assays. SB 203580 inhibited its established targets, SAPK2a/p38 and SAPK2b/p38β2 (Table 1), with IC_{50} values of 50 nM and 500 nM respectively in our standard assay [11]. LCK, GSK3β and PKBα were also inhibited by SB 203580 (Table 1), but the IC_{50} values were 100–500-fold higher than that for SAPK2a/p38 (results not shown). No other protein kinase in the panel was inhibited at a concentration (10 μM) which obliterates SAPK2a/p38 activity in cell-based assays (however, see the Discussion).

SB 202190

This compound is structurally very similar to SB 203580 and is sometimes used instead of SB 203580 to investigate potential roles for SAPK2a/p38 *in vivo*. However, to our knowledge, its specificity towards a wide range of protein kinases has never been examined. In the present study, we confirmed that SB 202190 has a specificity similar to that of SB 203580. It inhibited SAPK2a/p38 and SAPK2b/p38β2 with IC_{50} values of 50 nM and 100 nM respectively (Table 2). This compares with IC_{50} values for SB 203580 of 50 nM and 500 nM for SAPK2a/p38 and SAPK2b/p38β2 respectively under the same conditions [11].

Inhibitors of PI 3-kinase

Wortmannin inhibits PI 3-kinase in cell-based assays at a concentration of 100 nM. However, wortmannin also inhibits other members of the PI 3-kinase superfamily, which includes some protein kinases. Moreover, wortmannin was originally reported to be an inhibitor of SmMLCK [30]. In the present study, SmMLCK was inhibited by wortmannin with an IC_{50} value of 260 nM, but the skeletal muscle isoform (SkMLCK) and

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Table 3 Concentration of LiCl required for 50% inhibition of protein kinases in the presence of different concentrations of Mg^{2+} and KCl

The ATP concentration was 0.1 mM in all assays.

Protein kinase	[Mg^{2+}] (mM)	[KCl] (mM)	IC_{50} (mM)
GSK3β	10	0	15
GSK3β	0.5	0	5
GSK3β	0.5	150	2
MAPKAP-K2	10	0	21
MAPKAP-K2	0.5	0	5
MAPKAP-K2	0.5	150	10
PRAK	10	0	56
PRAK	0.5	0	13
PRAK	0.5	150	17
CK2	10	0	44
CK2	0.5	0	13
CK2	0.5	150	7

other protein kinases in our panel were unaffected by 1 μM wortmannin (Table 1).

LY 294002 is another commonly used, but much less potent, inhibitor of PI 3-kinase, which also inhibits other members of the PI 3-kinase superfamily. Surprisingly, we found that LY 294002 inhibited CK2 with a potency similar to that for PI 3-kinase. LY 294002 was a much weaker inhibitor of SmMLCK than was wortmannin; conversely, wortmannin did not inhibit CK2.

Quercetin is marketed by Calbiochem as a PI 3-kinase inhibitor. However, we found that a number of protein serine/threonine kinases (e.g. AMPK, CK2, MAPKAP-K1/RSK2 and S6K1) were inhibited with a potency similar to that for PI 3-kinase.

Rapamycin

No protein kinase tested was inhibited significantly by rapamycin at 1 μM, a concentration 10–20-fold higher than that required to inhibit mTOR in cell-based assays.

Lithium ions

Lithium ions have been reported to inhibit GSK3β, but not several other protein kinases tested [31], and to mimic cellular effects thought to be mediated by the inhibition of this enzyme. These include the insulin-induced activation of glycogen synthase [32] and Wingless signalling in *Drosophila* [31].

Li^+ was found to inhibit GSK3β more potently than any other protein kinase in the panel, although several kinases were inhibited under conditions where the equivalent concentration of K^+ had no effect (Table 1). Li^+ became a more potent inhibitor of GSK3β and other protein kinases if the concentration of Mg^{2+} was decreased from 10 mM to 0.5 mM, a level thought to equate to the free concentration of Mg^{2+} in many cells. At 0.5 mM Mg^{2+} and isotonic KCl (0.15 M), GSK3β was inhibited by Li^+ (IC_{50} 2 mM) at a 3–7-fold lower concentration than were CK2, PRAK and MAPKAP-K2 (Table 3). Since CK2 phosphorylates glycogen synthase, and since phosphorylation by CK2 is a prerequisite for phosphorylation of glycogen synthase by GSK3

Table 4 Effects of Ro 318220 and other bisindoylmaleimides on the activities of protein kinases

The inhibitor concentrations used are shown in parentheses. Results are presented as kinase activity as a percentage of that in control incubations (averages of duplicate determinations). ATP was present at 0.1 mM in all assays. Ro 318220 also had no effect on MKK3, MKK4, MKK6 and MKK7.

	Activity (% of control)										
Protein kinase	Ro 318220 (1 μM)	Bis-1 (1 μM)	Bis-3 (1 μM)	Bis-4 (1 μM)	Bis-5 (1 μM)	Bis-8 (1 μM)	Bis-10 (1 μM)	Go6976 (1 μM)	K252c (10 μM)	KT 5720 (10 μM)	UCN1 (1 μM)
MKK1	94±1	94±1	90±1	84±0	99±0	99±1	94±1	64±1	59±0	20±1	90±3
MAPK2/ERK2	97±2	98±14	107±3	86±6	101±0	105±19	106±2	34±5	37±1	57±3	87±5
JNK1α1/SAPK1c	95±1	91±0	100±6	95±1	100±1	95±8	103±8	88±2	82±4	81±0	98±2
SAPK2a/p38	84±8	88±4	104±2	90±6	101±7	115±2	117±0	92±5	100±18	103±1	92±1
SAPK2b/p38β2	97±1	92±14	115±23	88±2	104±12	104±5	102±9	81±7	88±4	98±0	91±13
SAPK3/p38γ	92±1	89±6	116±4	83±2	99±4	107±17	116±7	72±3	64±1	95±2	76±1
SAPK4/p38δ	104±3	113±9	133±3	90±13	88±5	129±11	112±3	96±2	93±0	95±2	91±6
MAPKAP-K1b	2±0	9±2	2±1	54±5	92±7	4±1	5±1	5±1	29±3	23±1	5±1
MAPKAP-K2	103±8	90±8	97±4	113±5	124±4	104±8	105±11	80±0	83±5	62±3	90±0
MSK1	2±1	21±1	9±2	56±4	103±15	13±0	5±1	2±1	62±6	39±8	19±1
PRAK	96±6	98±10	89±8	96±15	85±1	102±5	101±11	76±10	50±3	58±8	77±7
PKA	70±2	99±2	87±2	93±5	95±8	94±2	90±3	85±5	99±0	39±5	39±1
PKCα	3±0	4±0	7±0	57±1	104±2	14±0	1±0	7±0	60±1	87±1	1±0
PDK1	84±9	85±2	69±2	95±8	109±9	107±8	122±3	18±0	6±0	7±1	0±0
PKBα	73±0	77±16	99±5	101±5	91±3	98±7	104±2	87±2	65±2	36±4	31±3
SGK	21±0	63±6	26±3	101±7	96±7	28±4	34±3	77±2	28±2	29±2	63±10
S6K1	6±0	32±8	18±1	87±4	110±1	29±2	12±1	12±1	49±3	66±2	28±2
GSK3β	5±0	50±5	46±4	49±4	109±5	21±6	42±3	33±2	84±0	39±3	83±1
ROCK-II	92±2	90±8	89±8	100±9	107±5	106±1	103±5	41±1	19±1	84±1	22±1
AMPK	42±0	54±0	23±1	96±3	102±1	60±0	71±4	50±0	30±0	22±1	1±0
CK2	104±5	101±2	106±2	99±3	98±1	103±5	104±4	101±1	67±2	95±7	77±7
PHK	57±2	54±2	20±0	90±3	100±4	58±0	60±2	1±1	0±0	0±0	0±0
LCK	79±0	86±3	86±2	91±4	96±8	86±1	98±8	58±3	68±1	46±6	7±0
CHK1	42±2	60±2	40±1	91±2	104±4	71±3	50±1	3±0	4±0	13±1	3±0
CHK2											69±1

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[33], it is not inconceivable that the activation of glycogen synthase by Li^+ in cell-based assays results from the inhibition of CK2, as well as of GSK3.

Ro 318220 and other bisindoylmaleimides

Ro 318220 was originally developed as a PKC inhibitor [34] and has been used in literally hundreds of studies to 'identify' physiological roles for this protein kinase. We have reported previously that Ro 318220 also inhibits MSK1, MAPKAP-K1/RSK and S6K1 with a potency similar to that for PKC *in vitro* [35,36]. In the present study, Ro 318220 was also found to be a potent inhibitor of GSK3 (Tables 2 and 4); a similar observation was made by others while this paper was in preparation [37]. It has been shown recently that Ro318220, in addition to inhibiting protein kinases, also directly inhibits voltage-dependent Na^+ channels [37a].

We tested many other bisindoylmaleimide derivatives that are marketed as PKC inhibitors. They all inhibited many protein kinases and, in all cases, S6K1, MAPKAP-K1/RSK2 and MSK1 were inhibited with a potency comparable with that for PKCα. Go6976 was also a potent inhibitor of CHK1 and PHK. Bis-5, a related compound that does not inhibit PKCs, did not inhibit any kinase in the panel (Table 4). A related inhibitor, K252c, which is marketed as an inhibitor of PKC and as a weaker inhibitor of PKA, had little or no effect on these kinases in our assays. It was a stronger inhibitor of CHK1, PDK1 and PHK (Table 4).

KT 5720, a compound related to K252c, is marketed as 'a potent specific inhibitor of PKA, which does not significantly inhibit PKC, PKG and MLCK' (PKG is cGMP-dependent protein kinase). However, we found that KT 5720 inhibited many protein kinases, and several were inhibited far more potently than PKA (Table 4). For example, PHK was inhibited 300 times more strongly than was PKA (Table 2).

UCN1 (7-hydroxystaurosporine) was originally described as an activator of cdc25, the protein phosphatase that stimulates cell cycle progression by activating cyclin-dependent protein kinases [38]. Subsequently it was reported to inhibit CHK1, one of the protein kinases that inactivates cdc25 to arrest the cell cycle when DNA is damaged [39]. In contrast, CHK2 (also called cds1), another cdc25-inactivating kinase activated in response to DNA damage, was not inhibited by UCN1 [39]. In the present study, we found that UCN1 inhibited five other protein kinases in our panel with a potency similar to that for CHK1 (Table 4). In our assays, the IC_{50} value for inhibition by UCN1 of CHK2 was 2.3 μM, compared with 2 nM for CHK1 (results not shown).

Chelerythrine and 10-[3-(1-piperazinyl)propyl]-2-trifluoromethyl-phenothiazine

These compounds are marketed as PKC inhibitors. However, they did not inhibit PKCα, or any other protein kinase, when used at a concentration of 10 μM in our assays (results not shown).

DISCUSSION

The work described in the present paper emphasizes the value of examining the specificities of protein kinase inhibitors against many members of this gene family. For example, KT 5720 was reported to be a specific inhibitor of PKA because much higher concentrations did not inhibit PKC, PKG or MLCK. However, the data presented in Table 4 show that KT 5720 inhibits many protein kinases and that some are inhibited far more potently than PKA. For example, PHK was inhibited 300-fold more potently than PKA (Table 2). Surprisingly, Rottlerin, which is marketed as a PKC inhibitor (especially of PKCδ), did not inhibit PKCα or PKCδ significantly in our assays, although it inhibited many protein kinases, especially MAPKAP-K2 and PRAK (Tables 1 and 2). Compounds such as KT 5720, Rottlerin and quercetin inhibit many protein kinases, and conclusions drawn about the role of particular protein kinases from their use are likely to be erroneous.

Although compounds that inhibit more than one protein kinase cannot be used to establish physiological roles for a particular enzyme, they can be useful in excluding the involvement of some protein kinases in the regulation of a cellular process. For example, stimuli that activate SAPK2a/p38 and/or the classical MAPK cascade activate the cAMP-response element binding protein (CREB) by phosphorylating it at Ser-133. The same stimuli also induce phosphorylation of the chromosomal proteins histone H3 and HMG14 (where HMG is high-mobility group) at Ser-10 and Ser-6 respectively. The phosphorylation of CREB, histone H3 and HMG14 is prevented by SB 203580, PD 98059 or a combination of these inhibitors. However SAPK2a/p38 or ERKs do not phosphorylate these proteins at the relevant sites *in vitro*, indicating that other protein kinases 'downstream' of SAPK2a/p38 and MAPKs/ERKs mediate these effects. Indeed, several such kinases have been shown to phosphorylate CREB at Ser-133 (MSK1, MAPKAP-K2), histone H3 at Ser-10 (MSK1, MAPKAP-K1) and HMG14 at Ser-6 (MSK1) [36,40,41]. However, only MSK1 and MAPKAP-K1 are inhibited by Ro 318220 and H89, whereas other protein kinases activated by SAPK2/p38 and/or MAPKs/ERKs [MAPKAP-K2, PRAK (Tables 1, 2 and 4), MAPK-integrating kinase (MNK1) and MNK2 (G. Scheper and C. G. Proud, personal communication)] are unaffected. Moreover, Ro 318220 and H89 do not inhibit the activation or activity of SAPK2a/p38 *in vitro* or in cell-based assays [35,36,42] (Tables 1 and 4). Therefore the finding that Ro 318220 and H89 inhibit the phosphorylation of CREB at Ser-133 in response to stimuli that activate SAPK2a/p38 [35,36,42] excludes the involvement of MAPKAP-K2, the closely related MAPKAP-K3, PRAK, MNK1 and MNK2. However, further information will be needed to establish whether MSK1 or MAPKAP-K1 mediates the phosphorylation of CREB at Ser-133 in response to agonists that activate the classical MAPK cascade, since both enzymes are activated by ERKs. Similarly, the phosphorylation of histone H3 at Ser-10 and of HMG14 at Ser-6 is prevented by H89 [40], indicating that phosphorylation is not catalysed by MAPKAP-K2, MAPKAP-K3, PRAK, MNK1 or MNK2. It was reported that H89 does not inhibit two isoforms of MAPKAP-K1/RSK *in vitro* [40], leading to the conclusion that MAPKAP-K1/RSK does not mediate the phosphorylation of histone H3 or HMG14 induced by agonists that stimulate the classical MAPK cascade. However, in the present study we found that MAPKAP-K1/RSK was inhibited by H89, although a 20-fold higher concentration was needed compared with MSK1 (Tables 1 and 2). Therefore more evidence is required to exclude the involvement of MAPKAP-K1/RSK. In particular, it would be necessary to show that H89 does not block the phosphorylation of authentic physiological substrates of MAPKAP-K1/RSK at a concentration that prevents the phosphorylation of histone H3 and HMG14. This is critical, because the EGF-induced phosphorylation of histone H3 is reported not to occur in human cells that lack the RSK2 isoform of MAPKAP-K1 [41], implying that histone H3 is a direct or indirect target of MAPKAP-K1/RSK2.

In contrast with results presented by Favata et al. [28], we found that U0126 prevents the activation of MKK1 in cell-based

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assays or *in vitro*, and at concentrations over 100-fold lower than those that inhibit MKK1 activity *in vitro* (Figure 1). Similarly, we found that the recently discovered MKK1 inhibitor PD 184352 blocks the MAPK cascade in cells by preventing the activation of MKK1, and not by blocking its activity [16] (Figure 2). The mechanism by which U0126 and PD 184352 exert their effects *in vivo* is therefore similar to that of PD 98059. U0126 inhibited a few other protein kinases in our panel, but only at concentrations nearly 1000-fold higher than those required to suppress the activation of MKK1 and the MAPK cascade in cells. PD 184352 did not inhibit other protein kinases in the panel (Table 1 and 2).

It was reported recently that U0126 and PD 98059 prevent the activation of ERK5, another MAPK family member that is activated by MKK5 in response to growth factors or oxidative stress. The concentrations of U0126 and PD 98059 needed to suppress the activation of ERK5 were similar to those required to prevent activation of ERK1/ERK2 [43]. Thus the cellular effects of PD 98059 and U0126 may result from the inhibition of either of two MAPK cascades. As found for MKK1, PD 98059 appears to exert its effects on the activation of ERK5 by preventing the activation of MKK5, and not by inhibiting its activity [43]. It will be interesting to see whether this is also true for U0126. It will also be interesting to ascertain whether PD 184352 inhibits the activation or activity of MKK5.

Although PD 98059 does not inhibit any protein kinase in our panel, it is known to inhibit cyclo-oxygenases 1 and 2, the rate-limiting enzymes in the production of prostaglandins and leukotrienes, and this underlies its ability to decrease platelet aggregation induced by low concentrations of arachidonic acid [44]. In contrast, U0126 does not inhibit cyclo-oxygenases 1 or 2 or platelet aggregation at concentrations that completely inhibit activation of the classical MAPK cascade (S. Watson, personal communication). It is therefore important to use both PD 98059 and U0126 in all cell-based assays to minimize the risk of studying non-specific effects. Similarly, in the present study, we found that the PI 3-kinase inhibitor LY 294002 inhibits the serine/threonine-specific protein kinase CK2 with similar potency. In contrast wortmannin, another inhibitor of PI 3-kinase, does not inhibit CK2. This again points to the importance of using at least two structurally unrelated inhibitors of any particular protein kinase, in order to minimize the chance that any observed effects in cell-based assays result from a non-specific effect of a drug.

Nearly all the protein kinase inhibitors that have been developed are ATP-competitive [1], although U0126, PD 98059, PD 184352 and Li^+ are exceptions. For this reason, drug concentrations required for 50 % inhibition (IC_{50}) usually depend on the ATP concentration used in the assays. For example, the Calbiochem catalogue reports that KT 5720 inhibits PKA with an IC_{50} of 56 nM, but these assays were conducted at the extremely low ATP concentration of 5 μM. In contrast, the IC_{50} value in our assay was 2–3 μM, which was conducted at 0.1 mM ATP. For these and other reasons, such as problems of cell permeability, the drug concentrations that are effective in inhibiting a particular protein kinase *in vivo* (where ATP concentrations are in the millimolar range) are usually far higher than those found to inhibit activity *in vitro*. Thus Ro 318220 inhibits PKC, MSK1 and other protein kinases at nanomolar concentrations *in vitro* (Table 2), but low micromolar concentrations are required for equivalent inhibition in cell-based assays [36,42]. However, the potency of inhibitors *in vivo* can be as high as (or higher than) that *in vitro* if they act in other ways. For example, U0126 and PD 184352 prevent the activation of MKK1 at concentrations that are much lower than those that suppress MKK1 activity (Figures 1 and 2). This suggests that the drug binds much more tightly to the inactive dephosphorylated form of MKK1 than to the active phosphorylated species. This may also be the case for some ATP-competitive inhibitors, such as SB 203580, which is reported to inhibit the activation of SAPK2a/p38 in cell-based assays [45]. These drugs may induce conformational changes in the inactive dephosphorylated protein kinase that prevent activation by the next 'upstream' kinase in the cascade.

A further important point that is clearly shown by the present study is that the specificity of a protein kinase inhibitor cannot be gauged simply by comparing its effects on the protein kinases to which it is most closely related. For example, Ro 318220 inhibits 'conventional' isoforms of PKC (PKCα, PKCβ and PKCγ) far more potently than the 'atypical' PKC isoforms (PKCϵ and PKCζ) and other members of the 'AGC' subfamily of protein kinases, which includes PKA and PKG. Nevertheless, Ro 318220 also inhibits GSK3, which is a member of a distinct protein kinase subfamily, with a potency similar to that for PKC (Tables 1 and 2; [37]). Similarly, SB 203580 inhibits SAPK2a/p38 and the closely related SAPK2b/p38β2 (collectively termed SAPK2/p38), but not SAPK3/p38γ or SAPK4/p38δ, the MAPK family members to which they are most closely related. The basis for the specificity of SB 203580 became clear when the three-dimensional structure of SAPK2a/p38 was solved in complex with closely related drugs [46,47]. These studies revealed that, in order to accommodate the 4-fluorophenyl moiety of SB 203580, the residue at position 106 must not be larger than threonine. The presence of larger side chains at this position explains the insensitivity of SAPK3/p38γ, SAPK4/p38δ and many other protein kinases to the drug. However, the few other protein kinases that possess threonine and serine at this position, such as Raf [48] and LCK (Table 1), are inhibited by SB 203580, albeit less potently than SAPK2a/p38 [11,48].

It has been reported that SB 203580 inhibits the protein kinase PDK1 *in vitro* and that this underlies the suppression by SB 203580 of interleukin-2-stimulated T-cell proliferation that is unrelated to inhibition of SAPK2/p38 [49]. However, we have failed to observe any inhibition of PDK1 by SB 203580 in our assays using PDKtide as substrate (Table 1), or with SGK, another of its physiological substrates (results not shown). In addition, we have reported (and confirmed in many subsequent experiments) that SB 203580 does not prevent the activation of PKB by insulin-like growth factor I, hydrogen peroxide or heat shock in Swiss 3T3 or HEK-293 cells [50]. The suppression of interleukin-2-induced T-cell proliferation by SB 203580 must therefore be explained by another mechanism.

Small cell-permeant inhibitors have several advantages for the study of cell signalling. First, they can be used simply and rapidly to assess the physiological roles of protein kinases, and in normal cells and tissues as opposed to transformed cell lines. Secondly, they inhibit the endogenous protein kinases, avoiding the need for overexpression of dominant-negative and constitutively active protein kinases, which can cause the specificity of signalling to break down and lead to erroneous conclusions being drawn. However, as is obvious from the above discussion, results obtained by the use of inhibitors are also dangerous because, even if a drug inhibits just one out of 20–30 protein kinases tested, there is no guarantee that it does not inhibit other protein kinases or other types of enzyme that have not been examined. In the case of SB 203580, we have recently addressed this criticism by inducibly expressing an SB 203580-resistant mutant of SAPK2a/p38. In cells expressing this mutant, SB 203580 no longer blocks the activation and/or phosphorylation of several putative downstream targets of SAPK2a/p38, establishing that

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they are indeed *in vivo* substrates [51]. In contrast, effects of SB 203580 that are not mediated by SAPK2a/p38, but result from the binding of this drug to another protein kinase, are unaffected by overexpression of the drug-resistant mutant [51]. Rapamycin-resistant mutants of TOR have been used in a similar way [52]. It will clearly be important to extend this approach to other protein kinase inhibitors.

Recommendations for the use of protein kinase inhibitors in cell-based assays

1. In order to validate the results, show that the effects of an inhibitor disappear when a drug-resistant mutant of the protein kinase is overexpressed.

2. If a drug-resistant mutant is not available, show that the cellular effect of the drug occurs at the same concentration that prevents the phosphorylation of an authentic physiological substrate of the protein kinase (measured in the same cell extract). Also, make sure that the same effect is observed with at least two structurally unrelated inhibitors of the protein kinase. Only use inhibitors whose specificities have been tested towards a wide range of protein kinases *in vitro*.

3. Most protein kinase inhibitors are ATP-competitive. Therefore remember that much higher drug concentrations are usually required to suppress the phosphorylation of a substrate in cells, as compared with those required for inhibition *in vitro*.

4. Relatively non-specific protein kinase inhibitors can still be useful for excluding the involvement of a particular protein kinase(s) in the control of a cellular process.

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