The effect of the farnesyl protein transferase inhibitor SCH66336 on isoprenylation and signalling by the prostacyclin receptor

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Like Ras, farnesylation of the IP (prostacyclin receptor) is required for its efficient intracellular signalling, and hence the IP represents a potential target for inhibition by FTIs [FTase (farnesyl protein transferase) inhibitors]. Herein, the effect of SCH66336 on the isoprenylation and function of the human and mouse IPs over-expressed in human embryonic kidney 293 cells, and by the IP endogenously expressed in human erythroleukaemia cells, was investigated. SCH66336 yielded concentration-dependent decreases in IP-mediated cAMP generation (IC_{50} 0.27–0.62 nM), $[Ca^{2+}]_i$ mobilization (IC_{50} 26.6–48.3 nM) and IP internalization, but had no effect on signalling by the non-isoprenylated β_2 adrenergic receptor or β isoform of the TP (prostanoid thromboxane A_2 receptor). Additionally, SCH66336 impaired IP-mediated cross-desensitization of TP α signalling (IC_{50} 56.1 nM) and reduced farnesylation of the molecular chaperone protein HDJ-2 (IC_{50} 3.1 nM). To establish whether farnesylation of the IP is inhibited

and/or whether its 'CaaX motif' might undergo alternative geranylgeranylation in the presence of SCH66336, a series of chimaeric Ha (Harvey)-Ras fusions were generated by replacing its CaaX motif (-CVLS) with that of the IP (-CSLC) or, as controls, of Ki (Kirsten)-Ras 4B (-CVIM) or Rac 1 (-CVLL). Whereas SCH66336 had no effect on Ha-Ras^{CVLL} isoprenylation *in vitro* or in whole cells, it supported alternative geranylgeranylation of Ha-Ras^{CVIM}, but completely impaired isoprenylation of both Ha-Ras^{CVLS} and Ha-Ras^{CSLC}. These data confirm that the -CSLC motif of the IP is a direct target for inhibition by the FTI SCH66336, and in the presence of strong FTase inhibition, the IP does not undergo compensatory geranylgeranylation.

Key words: desensitization, farnesyl protein transferase inhibitor, isoprenylation, prostacyclin receptor, Ras, thromboxane A_2 .

INTRODUCTION

Ras guanine-nucleotide-binding proteins act as molecular switches, transducing an array of extracellular growth and differentiation signals from cell-surface receptors to the nucleus [1]. Mutations in codon 12, 13 or 61 of one of the three *ras* genes, i.e. Ha (Harvey)-*ras*, N (neuronal)-*ras* or Ki (Kirsten)-*ras*, encode a constitutively activated protein that transforms cells to a malignant phenotype by providing uninterrupted growth signals [2,3]. In mammalian cells, Ras becomes biologically active following a series of post-translational modifications, which allow for its subsequent localization to the inner face of the plasma membrane [4]. The first modification is initiated by the formation of a stable thioether bond between a cysteine located in a distinct C-terminal motif (CaaX) and a hydrophobic isoprenyl (farnesyl or geranylgeranyl) group derived from their pyrophosphate intermediates of the cholesterol biosynthetic pathway [4]. Farnesylation, catalysed by FTase (farnesyl protein transferase), is directed by a C-terminal CaaX motif, where 'C' is cysteine, 'a' is usually an aliphatic residue, and 'X' is typically methionine, serine, glutamine, cysteine or alanine. Where 'X' represents a leucine or isoleucine, the protein becomes geranylgeranylated, catalysed by GGase 1 (geranylgeranyl protein transferase 1) [4]. Proteolytic cleavage to remove the 'aaX' residues, followed by carboxyl-methylation of the farnesyl/geranylgeranyl-cysteine residue, completes the CaaX-directed series of post-translational modifications [4].

Given that oncogenic mutations of the *ras* gene occurs in over 30 % of all known human cancers, the Ras signalling pathway represents a major therapeutic target in malignant transformation [1]. Indeed, the improved understanding of the molecular mechanisms of Ras expression, processing, activation and action on downstream effectors has promulgated the development of novel agents designed to target this critical aberrant pathway [2]. Of these, the FTIs (FTase inhibitors) have shown most potential, having reached phase III clinical trial status [5]. By inhibiting farnesylation of Ras, FTIs prevent its subsequent obligate localization to the plasma membrane, and hence impair transduction of its proliferative signals [6].

SCH66336 (Ionafarnib[®]; Sarasar[®]) was the first FTI to undergo clinical development [5]. A non-peptidyl, non-thiol tricyclic FTI, SCH66336 has demonstrated significant biological and clinical activity in a range of malignancies and solid tumours [5,6]. Confirmation of biological efficacy has been demonstrated further with surrogate markers in various clinical trials: SCH66336 inhibited prelamin A farnesylation in buccal mucosa cells at clinically relevant doses, while an 11–50 % increase in non-farnesylated molecular chaperone protein HDJ-2 was observed in surgical specimens from patients treated with 100, 200 or 300 mg SCH66336 twice daily for 8–14 days [5].

Although SCH66336 is selective in *in vitro* assays, there is currently insufficient published data to confirm that FTIs do not interfere, adversely or otherwise, with farnesylation and function of other unidentified proteins. In the present study, we have

Abbreviations used: β_2 AR, β_2 adrenergic receptor; $[Ca^{2+}]_i$, intracellular calcium; FBS, fetal bovine serum; FPP, farnesyl pyrophosphate; FTase, farnesyl protein transferase; FTI, farnesyl protein transferase inhibitor; GGPP, geranylgeranyl pyrophosphate; fura 2/AM, fura 2 acetoxymethyl ester; GGase, geranylgeranyl protein transferase; GPCR, G-protein-coupled receptor; HA, haemagglutinin; Ha-Ras, Harvey Ras; HEK, human embryonic kidney; HEL, human erythroleukaemia; (h/m)IP, (human/mouse) prostacyclin receptor; Ki-Ras, Kirsten Ras; LDH, lactate dehydrogenase; MVA, mevalonolactone; N-Ras, neuronal Ras; PKA, cAMP-dependent protein kinase; PLC β , phospholipase C β ; TXA $_2$, thromboxane A_2 ; TP, prostanoid TXA $_2$ receptor.

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examined the effect of SCH66336 on farnesylation of one such protein, the IP (prostacyclin receptor). IP mediates the action of prostacyclin [prostaglandin (PG)_{I2}], a labile cyclo-oxygenase-derived metabolite of arachidonic acid [7]. Prostacyclin plays a prominent role in vascular haemostasis, acting as a potent inhibitor of platelet aggregation and as a vasodilator [7]. Perturbations in prostacyclin and/or IP signalling have been implicated in the pathogenesis of a number of disorders, including ischaemic heart disease, atherosclerosis, renal failure and systemic and pregnancy-induced hypertension [7–10]. The finding that both the human (h) and mouse (m) IP are farnesylated, and that this modification is absolutely required for efficient receptor-effector coupling, raises the possibility that SCH66336 may inadvertently interfere with IP signalling, and hence IP function [11,12]. Thus the overall aim of the present study was to investigate the effect of SCH66336 on farnesylation and intracellular signalling by hIP and mIP expressed in mammalian cells with the view to ascertaining whether the IP is a likely therapeutic target for FTI inhibition, and to assess the potential implications thereof. In addition, we sought to examine whether the 'CAAX' motif of the IP is solely a substrate for farnesylation, or whether it undergoes alternative geranylgeranylation in the presence of SCH66336.

EXPERIMENTAL

Materials

SCH66336 was obtained from Schering Plough Inc. (Kenilworth, NJ, U.S.A.). Cicaprost was obtained from Schering AG (Berlin, Germany). Iloprost, [³H]iloprost (15.3 Ci · mmol⁻¹), [³H]CGP-12177 (41.0 Ci · mmol⁻¹) and PVDF filters were purchased from Amersham Biosciences. [³H]MVA ([³H]mevalonolactone; 20 Ci · mmol⁻¹), [³H]farnesylpyrophosphate (20 Ci · mmol⁻¹), [³H]geranylgeranylpyrophosphate (15–30 Ci · mmol⁻¹), [³⁵S]methionine (1175 Ci · mmol⁻¹) and [³H]cAMP (15–30 Ci · mmol⁻¹) were purchased from American Radiolabeled Chemicals Inc. Isoproterenol was purchased from Sigma. Fura 2/AM (fura 2 acetoxymethyl ester) and U46619 was purchased from Calbiochem. Rabbit reticulocyte translation system (minus methionine), T7 RNA polymerase, RNasin and all restriction endonucleases were purchased from Promega Corp. *Taq* DNA polymerase, the chemiluminescence Western detection kit, and rat monoclonal 3F10 anti-HA (haemagglutinin) peroxidase-conjugated antibody were purchased from Roche. Horseradish peroxidase-conjugated goat anti-mouse IgG was from Santa Cruz Biotechnology. Mouse monoclonal 101R anti-HA antibody was obtained from BabCO. Anti-HDJ2 was from Neomarkers. Secondary FITC-conjugated anti-mouse IgG serum was purchased from Jackson ImmunoResearch Laboratories, Inc. Oligonucleotides were synthesized by Genosys Biotechnologies. All other reagents were A. R. or molecular biology grade, and were used without further purification.

Cell culture and transfections

Human embryonic kidney (HEK).mIP, HEK.hIP, HEK.TP α [where 'TP α ' is prostanoid thromboxane A₂ (TXA₂) receptor] and HEK.TP β cells, stably overexpressing HA-epitope-tagged forms of the wild-type mIP and hIP or the human TP α and β isoforms respectively, have been described previously [11,13]. HEK-293 cells were transiently transfected with pHM- or pCMV-based vectors using the calcium phosphate/DNA co-precipitation procedure [11,13] and were harvested 48 h post-transfection.

To generate a mammalian cell line overexpressing the β_2 AR (β_2 adrenergic receptor), a *HindIII*–*EcoRI* subfragment encod-

ing the full-length cDNA for the human β_2 AR was subcloned from the plasmid pTF3 (A.T.C.C.) into a *HindIII*–*EcoRI* site of pHM6 (Roche) to generate pHM: β_2 AR. Thereafter, HEK. β_2 AR cell lines stably overexpressing the β_2 AR receptor in HEK-293 cells were established, using pHM: β_2 AR, and characterized essentially as described previously [11,13].

Subcloning and site-directed mutagenesis of the 'CaaX motif' of Ras

The plasmid pHM:Ras was constructed by subcloning the full-length coding sequence for wild-type human Ha-Ras (Ha-Ras^{WT}) from pCMV5:Ras [14] into the *HindIII* site of pHM6 (Roche). To generate Ha-Ras^{CSLC}, the C-terminal CaaX motif of that of Ha-Ras^{WT} (with the sequence Ha-Ras^{CVLS}) was mutated by site-directed mutagenesis to that of the IP (with the sequence IP^{CSLC}). Specifically, site-directed mutagenesis of Val¹⁸⁷ and Ser¹⁸⁹ of Ha-Ras^{WT} to Ser¹⁸⁷ and Cys¹⁸⁹ respectively to generate Ha-Ras^{CSLC} was performed using the following mutator oligonucleotide: 5'-dCTC AAG CT TCA GCA GAG CGA ACA CTT GCA GCT CAT GC-3' (antisense primer; the sequence complementary to the mutator bases are highlighted in boldface italics). Similarly, to generate Ha-Ras^{SSLC}, Cys¹⁸⁶, Val¹⁸⁷ and Ser¹⁸⁹ of Ha-Ras^{WT} were converted into Ser¹⁸⁶, Ser¹⁸⁷ and Cys¹⁸⁹ respectively using the mutator oligonucleotide 5'-dCTC AAG CT TCA GCA GAG CGA AGA CTT GCA GCT CAT GCA GC-3' (antisense primer; the sequence complementary to the mutator bases are highlighted in boldface italic). To generate Ha-Ras^{CVLL}, Ser¹⁸⁹ of Ha-Ras^{WT} was converted into Leu¹⁸⁹ using the mutator oligonucleotide 5'-dCTC AAG CT TCA GAG GAG CAC ACA CTT GCA GC-3' (antisense primer). Finally, to generate Ha-Ras^{CVIM}, Leu¹⁸⁸ and Ser¹⁸⁹ of Ha-Ras^{WT} were converted into Ile¹⁸⁸ and Met¹⁸⁹ respectively, using the mutator oligonucleotide 5'-dAGA GGT ACC TTA CAT AAT CAC ACA CTT GCA GCT-3' (antisense primer).

In each case, PCR mutagenesis was performed using the oligonucleotide 5'-dGAG AAG CTT G ATG ACG GAA TAT AAG CTG GTG-3' as sense primer (whereby the sequence corresponding to the initiation codon is underlined) in combination with the specified mutator oligonucleotide, as antisense primer, using the plasmid pCMV:Ras [14] as a template. Thereafter, PCR-generated products were subcloned in-frame into the *HindIII* site of pHM6 to generate the plasmids pHM:Ras^{CSLC}, pHM:Ras^{SSLC}, pHM:Ras^{CVLL} or, in the case of Ras^{CVIM}, into *HindIII*–*KpnI* sites of pHM6 to generate the plasmid pHM:Ras^{CVIM}. All resulting plasmids were verified by double-stranded DNA sequence analysis and encode HA-epitope-tagged forms of Ha-Ras^{WT}, Ha-Ras^{CSLC}, Ha-Ras^{SSLC}, Ha-Ras^{CVLL} and Ha-Ras^{CVIM} respectively.

Radioligand binding studies

[³H]Iloprost radioligand binding assays of the IP were performed on cell membranes, as described previously [11]. β_2 AR and TP radioligand assays were performed on whole cells, as described previously [13,15]. Where specified, cells were pre-incubated with either 1 μ M SCH66336 (added from 1000-fold-concentrated stocks diluted in 0.0001 % DMSO) or with the corresponding volume of vehicle (DMSO) for 16 h before harvesting.

Measurement of SCH66336-induced cytotoxicity

Possible cytotoxic effects of SCH66336 were evaluated by measuring LDH (lactate dehydrogenase) release following exposure of cells for 24 h with 1 nM, 20 nM and 1000 nM SCH66336 using a Cytotox 96 Non-Radioactive Cytotoxicity assay kit (Promega), essentially as described by the manufacturer.

The percentage of LDH released from the cells was determined by expressing the level of SCH66336-induced LDH released as a fractional percentage of the maximum LDH release (i.e. % release LDH activity in SCH66336-treated cells/maximum LDH activity in cell lysate).

Measurement of cAMP

To investigate the effect of SCH66336 on agonist-mediated cAMP generation, cells were pre-incubated in the presence or absence of SCH66336 (10 nM) for 16 h before harvesting. For concentration–response studies, cells were pre-incubated for 16 h in the presence of SCH66336 (0–10 nM). For time-course assays, cells were pre-incubated for 0–24 h in the presence of SCH66336 (5 nM). Thereafter, non-viable cells were removed, and cAMP assays were performed as described previously [11].

Measurement of $[Ca^{2+}]_i$ (intracellular $[Ca^{2+}]_i$) mobilization

Measurements of $[Ca^{2+}]_i$ mobilization in fura 2/AM-preloaded cells was carried out essentially described as previously [11]. Cells were pre-incubated for 16 h with either SCH66336 (1000 nM) or with the vehicle, 0.0001 % DMSO. For concentration–response studies, cells were pre-incubated with SCH66336 (0–1000 nM) for 16 h. In separate experiments, to examine the concentration effect of SCH66336 on IP-mediated counter-regulation of TP-mediated signalling, HEK.TP α cells and HEK.TP β cells, transiently transfected with pCMV:G α_q , were pre-incubated in the presence or absence of SCH66336 (1000 nM) for 16 h. For concentration–response studies, cells were pre-incubated with SCH66336 (0–1000 nM) for 16 h. Thereafter, $[Ca^{2+}]_i$ mobilization in fura 2/AM-preloaded cells was measured essentially as described previously [11].

Immunofluorescence microscopy

Immunofluorescence analysis was performed essentially as described previously [12]. Cells were pre-incubated for 24 h with either SCH66336 (1000 nM) or with the vehicle, 0.0001 % DMSO. Thereafter, cells were stimulated with cicaprost (1 μ M; 4 h) or with vehicle (Tris-buffered saline), serving as a control. Immunofluorescent images, captured with the appropriate FITC filter, were monitored using an Axioplan 2 Fluorescent Microscope (Zeiss Vision GmbH, Cologne, Germany).

Agonist-induced IP internalizations

For quantification of agonist-induced IP internalization, ELISAs were performed essentially as described previously [12]. Cells were incubated with 1 μ M SCH66336 or with vehicle (0.0001 % DMSO) for 24 h. Internalizations of HA-tagged IP receptors were quantified as the mean percentage (%) of cell-surface IP in agonist-treated cells relative to vehicle (non-treated) cells $\pm$ S.E.M., where control, vehicle-treated cells were assigned a value of 100 %.

Immunoblot analysis of HDJ-2 isoprenylation

To investigate the effect of SCH66336 on HDJ-2 isoprenylation, cells were incubated with SCH66336 (0–10 nM) or with the vehicle (0.0001 % DMSO) for 24 h. For time-dependent-response studies, cells were incubated with SCH66336 (5 nM) or with the vehicle (0.0001 % DMSO) for 24 h. Thereafter, cells were harvested and aliquots of whole-cell protein (25 μ g) were analysed for HDJ-2 expression essentially as described previously [16]. Quantification of farnesylated and non-farnesylated HDJ-2 protein fractions was performed by densitometric scanning using a GelWorks 1D Intermediate UVP gel documentation system.

Percentage inhibition of HDJ-2 farnesylation was determined by comparing the signal in the non-farnesylated band with the overall intensities of farnesylated plus non-farnesylated bands. The levels of non-farnesylated protein in vehicle-treated cells were assigned a value of zero.

In vitro transcription, translation and isoprenylation

Prior to *in vitro* transcription, pHM:Ras^{WT}, pHM:Ras^{CSLC}, pHM:Ras^{SSLC}, pHM:Ras^{CVLL} and pHM:Ras^{CVIM} plasmids were linearized by digestion with *Eco*RI. The linearized plasmids (4 μ g) were transcribed with T7 RNA polymerase as previously described [14]. *In vitro* translation of the RNA transcripts was performed in a methionine-deficient rabbit reticulocyte lysate system (Promega) according to manufacturer's recommendations. Standard reactions (25 μ l) containing 2.5 μ g of RNA and either 20 μ Ci of L-[³⁵S]methionine, 25 μ Ci of [³H]MVA, 1.25 μ Ci of [³H]FPP (farnesyl pyrophosphate) or 1.25 μ Ci [³H]GGPP (geranylgeranyl pyrophosphate) were translated essentially as described previously [14]. To investigate the effect of SCH66336 on isoprenylation of the CAAX motif *in vitro*, RNA transcripts were translated in the presence of [³H]MVA $\pm$ SCH66336 (25 nM), [³H]FPP $\pm$ SCH66336 (25 nM) or [³H]GGPP $\pm$ SCH66336 (25 nM), as described previously [14]. Proteins from each reaction were subjected to SDS/PAGE, followed by electroblotting on to PVDF membranes. Blots were soaked in Amplify (Amersham Biosciences) for 30 min, followed by autoradiography using Kodak Xomat XAR film at -70°C for at least 21 days for ³H exposures, or for 16 h for [³⁵S]methionine exposures.

In vivo isoprenylation in whole cells

To investigate the ability of SCH66336 to interfere with isoprenylation of Ha-Ras^{WT}, Ha-Ras^{CSLC}, Ha-Ras^{SSLC}, Ha-Ras^{CVLL} and Ha-Ras^{CVIM} *in vivo*, metabolic labelling of whole cells was performed essentially as described previously [11]. Cells were pre-incubated for 16 h with either 250 μ Ci [³H]MVA plus 1 μ M SCH66336, or with 250 μ Ci [³H]MVA plus an equivalent volume of the drug vehicle (0.0001 % DMSO). Thereafter, cells were harvested and aliquots of whole cell protein (60 μ g) were resolved by SDS/PAGE and electroblotted on to PVDF membranes. The remaining protein from each transfection ($\approx$ 500 μ g) was subjected to immunoprecipitation using the anti-(HA 101R) serum, as described previously [11]. Blots were then permeated in Amplify (Amersham Biosciences) and subjected to fluorography, as described previously [11].

Data analysis

Statistical analysis was carried out using the unpaired Student's *t* test using the GraphPad Prism V2.0 program (GraphPad Software Inc., San Diego, CA, U.S.A.). *P* values $\leq$ 0.05 were considered to indicate a statistically significant difference.

RESULTS

SCH66336 is a selective FTase inhibitor undergoing clinical trials as a chemotherapeutic agent for the treatment of a variety of solid tumours [5] but, to date, has not yet been extensively tested against certain other proteins known to undergo farnesylation, such as the IP. Thus in the present study we investigated the potential effects of SCH66336 on isoprenylation and function of hIP and mIP stably expressed in HEK-293 cells, or by the hIP endogenously expressed in HEL92.1.7 ('HEL', human erythroleukaemia) cells.

Initially, the potential effect of SCH66336 on the cell viability of HEK-293 and HEL cells cultured in a range of SCH66336 concentrations was evaluated. While the level of SCH66336-induced

Table 1 Cytotoxic effects of SCH66336

HEK-293 and HEL92.1.7 (HEL) cells were pre-incubated with SCH66336 (1 nM, 20 nM or 1000 nM) or, as controls, with the drug-vehicle [0.0001% (v/v) DMSO] for 24 h. SCH66336-induced cytotoxicity was determined by measuring LDH release, and was expressed as a percentage of maximum LDH release determined in the presence of Triton X-100 (% cytotoxicity), as outlined in the Experimental section.

Cell type	[SCH66336] (nM) ...	Percentage cytotoxicity (%)		
		1	20	1000
HEK-293		2.6 ± 0.8	8.2 ± 1.4	18.6 ± 2.4
HEL		2.2 ± 0.4	5.5 ± 0.2	25.6 ± 2.1

cell cytotoxicity was low, it became significant at 20 nM (Table 1), with $75.2 \pm 7.5\%$ of HEK-293 cells and $75.2 \pm 1.4\%$ of HEL cells remaining viable following 24 h exposure to 1000 nM SCH66336. Similar data were generated in the clonal HEK.hIP and HEK.mIP cell lines stably overexpressing the HA-tagged forms of hIP and mIP respectively (results not shown). Hence, for all subsequent studies, measurement of ligand binding and intracellular signalling by the IPs was performed on viable cells only.

Thereafter, the effect of SCH66336 on intracellular signalling by the IP expressed in HEK.hIP and HEK.mIP cells, and in HEL cells was investigated by examining its effect on agonist-induced cAMP generation in response to the selective IP agonist cicaprost [7]. As a control, the effect of SCH66336 on cAMP generation by the non-isoprenylated G_i /adenylate cyclase-coupled β_2 AR in response to stimulation of HEK. β_2 AR cells with isoproterenol was also examined. Whereas HEK.hIP and HEK.mIP cells exhibited significant cicaprost-induced increases in cAMP, pre-incubation of those cells with 10 nM SCH66336 for 16 h reduced cAMP generation to levels that were not significantly different from those generated by the non-transfected parental HEK-293 cells (Figure 1A). From concentration–response studies, IC_{50} values for SCH66336 in HEK.hIP (Figure 1B) and HEK.mIP (Figure 1C) cells were determined to be 0.27 nM and 0.43 nM respectively, while the extent of maximal inhibition at 10 nM SCH66336 was found to be $71.4 \pm 7.1\%$ ($P \leq 0.0001$) and $74.4 \pm 9.4\%$ ($P \leq 0.0001$) for hIP and mIP respectively (Figures 1A–1C). Consistent with that observed in the HEK.hIP and HEK.mIP cell lines, agonist stimulation of hIP endogenously expressed in HEL cells exhibited significant fold increases in cicaprost-induced cAMP generation over basal levels (Figure 1A). Moreover, pre-incubation of HEL cells with SCH66336 (16 h) significantly reduced cicaprost-induced cAMP generation in a concentration-dependent manner (IC_{50} 0.62 nM; Figures 1A and 1D). From concentration–response studies, an IC_{50} of 0.62 nM SCH66336 was determined, while the extent of maximal inhibition of hIP signalling at 10 nM SCH66336 was $80.3 \pm 1.2\%$ (Figures 1A and 1D; $P \leq 0.0001$). Whereas stimulation of the control HEK. β_2 AR cells with isoproterenol (10 μ M) produced a 6.9 ± 0.21 -fold increase in cAMP generation over basal levels, in contrast with those studies involving the IPs, pre-treatment of HEK. β_2 AR cells with SCH66336 did not significantly affect the levels of isoproterenol-induced cAMP generation throughout the range of SCH66336 concentrations utilized (Figures 1A and 1E; $P \geq 0.5$).

The effect of SCH66336 on IP-mediated $[Ca^{2+}]_i$ mobilization

Although the IP is mainly coupled to G_s /adenylate cyclase activation, it also exhibits efficient coupling to G_q /PLC β (phospholipase C β) activation, with concomitant rises in $[Ca^{2+}]_i$ in certain cell types [11,12]. Hence, in the present study, we extended our

investigations to examine the effect of SCH66336 on cicaprost-induced $[Ca^{2+}]_i$ mobilization by hIP and mIP stably overexpressed in HEK-293 cells and in HEL cells. As a control for a non-isoprenylated G_q /PLC β -coupled GPCR (G-protein-coupled receptor), the effect of SCH66336 on agonist-induced $[Ca^{2+}]_i$ mobilization by the β isoform of TP (TP β) was investigated. Consistent with previous reports [11,12,15], stimulation of both HEK.hIP (Figure 2A; $\Delta[Ca^{2+}]_i = 130 \pm 3.54$ nM) and HEK.mIP (Figure 2C; $\Delta[Ca^{2+}]_i = 157 \pm 17.4$ nM) cells showed efficient $[Ca^{2+}]_i$ mobilization in response to the IP agonist cicaprost (1 μ M). Pre-incubation of cells with SCH66336 (1 μ M for 16 h) significantly reduced cicaprost-induced $[Ca^{2+}]_i$ mobilization by the hIP (Figure 2A; $\Delta[Ca^{2+}]_i = 48.3 \pm 4.0$ nM, $p \leq 0.0001$) and the mIP (Figure 2C; $\Delta[Ca^{2+}]_i = 59.7 \pm 3.2$ nM, $P = 0.0006$). The IC_{50} for inhibition of cicaprost-mediated signalling in HEK.hIP cells and HEK.mIP cells was 34.6 nM SCH66336 (Figure 2B) and 26.6 nM SCH66336 (Figure 2D), respectively. Moreover, the extent of maximal inhibition using 1 μ M SCH66336 was $62.9 \pm 3.0\%$ and $62.0 \pm 5.5\%$ for the hIP and mIP respectively, relative to that mobilized in the absence of SCH66336 (Figures 2A and 2C). Stimulation of the hIP endogenously expressed in HEL cells also exhibited efficient cicaprost-induced $[Ca^{2+}]_i$ mobilization (Figure 2E; $\Delta[Ca^{2+}]_i = 98.5 \pm 4.5$ nM), consistent with hIP coupling to G_q in megakaryocytic lineages, as reported previously [15]. Pre-incubation of HEL cells with SCH66336 (1 μ M for 16 h) significantly reduced cicaprost-induced $[Ca^{2+}]_i$ mobilization (Figure 2E; $\Delta[Ca^{2+}]_i = 29.6 \pm 12.6$ nM, $P = 0.0354$) in a concentration-dependent manner ($IC_{50} = 43.7$ nM; Figure 2F), whereas the extent of maximal inhibition observed at 1 μ M SCH66336 was $73.2 \pm 7.1\%$ (Figure 2E). In contrast, while stimulation of the control HEK.TP β cells, transiently co-transfected with G_q , showed efficient $[Ca^{2+}]_i$ mobilization in response to the TXA₂ mimetic U46619 (1 μ M; Figure 2G, $\Delta[Ca^{2+}]_i = 167 \pm 6.9$ nM), pre-incubation of those cells with SCH66336 (1 μ M for 16 h) did not significantly reduce U46619-mediated $[Ca^{2+}]_i$ mobilization (Figure 2H; $\Delta[Ca^{2+}]_i = 157 \pm 5.5$ nM, $P = 0.3535$).

The effect of SCH66336 on ligand binding properties of the IP

To exclude the possibility that the observed reductions in IP-mediated signalling might be indirect, such as due to an overall reduction in the level of IP expression rather than due to a functional impairment of IP coupling to G_s /adenylate cyclase or G_q /PLC β , the effect of SCH66336 on the radioligand binding properties of the hIP and mIP was investigated (Table 2). Pre-incubation of cells with SCH66336 (1 μ M for 16 h) did not significantly affect [³H]iloprost binding by the IPs either stably overexpressed in HEK-293 cells or in HEL cells (Table 2). Consistent with this, [³H]CGP-12177 binding by the β_2 AR and [³H]SQ29, 548 binding by the TP α or, indeed, the TP β isoform of the TP overexpressed in HEK-293 cells was not significantly impaired by SCH66336 (1 μ M, 16 h), being directly comparable with those levels observed in the absence of SCH66336 (Table 2).

The effect of SCH66336 on IP agonist-mediated internalization

The IP has been widely reported to undergo rapid agonist-induced internalization and desensitization following activation, and such desensitizations are believed to represent important mechanisms whereby the responses to the potent autocoid prostacyclin is dynamically regulated [17]. Through the generation of an isoprenylation-defective hIP^{SSLC}, we have recently established that, while both the hIP and hIP^{SSLC} undergo agonist-induced internalization, the kinetics and extent of hIP^{SSLC} internalization was impaired

Figure 1 Effect of SCH66336 on IP-mediated cAMP generation

HEK.hIP cells (hIP), HEK.mIP cells (mIP), HEL cells (HEL), HEK-293 cells or, as controls, HEK.β₂AR cells (β₂AR) were pre-incubated with (+) or without (–) 10 nM SCH66336 for 16 h before harvesting (A). HEK.hIP cells (hIP; B), HEK.mIP cells (mIP; C), HEL cells (HEL; D) or HEK.β₂AR cells (β₂AR; E) were pre-incubated with 0–10 nM SCH66336 for 16 h before harvesting. Thereafter, cells were stimulated with 1 μM cicaprost or, in the case of the HEK.β₂AR cells, with 10 μM isoproterenol. In each case, basal cAMP levels were determined by exposing the cells to the vehicle HBS under identical incubation conditions. Levels of cAMP produced in ligand-stimulated cells, relative to basal cAMP levels, in vehicle-treated cells were expressed as fold stimulation of basal (fold increase in cAMP ± S.E.M., *n* = 4). The asterisks (*) in (A) indicate that the level of cicaprost-mediated cAMP generation was significantly reduced in the presence of SCH66336 compared with vehicle-treated cells, where *** indicates *P* ≤ 0.001. Basal levels of cAMP in HEK.hIP, HEK.mIP, HEL, HEK-293 and in HEK.β₂AR cells were 0.57 ± 0.07, 0.22 ± 0.01, 0.99 ± 0.09, 0.41 ± 0.06 and 0.36 ± 0.03 nmol/mg of cell protein respectively.

relative to that of wild-type hIP, implying that farnesylation of the IP is also required for efficient agonist-induced IP desensitization and internalization [12]. Thus, in the current study, the possible effects of SCH66336 on cicaprost-induced IP internalization were investigated, as assessed by indirect immunofluorescence microscopy and ELISA. Initially, immunofluorescence microscopy was performed on HEK.hIP cells and, as a control, on HEK-293 cells under non-permeabilizing conditions (Figure 3A). The hIP was abundantly expressed on the plasma membrane of HEK.hIP cells, as evidenced by strong immunostaining of cells (Figure 3Ai). In contrast, the control HEK-293 cells showed no immunofluorescent staining (Figure 3Aiv). Consistent with previous reports [12], the hIP underwent cicaprost-induced (1 μM for 4 h) internalization, as evidenced by a decrease in surface expression of the HA-epitope-tagged hIP

compared with that of vehicle-treated cells (Figure 3Aii). However, pre-incubation of HEK.hIP cells with SCH66336 (1 μM for 24 h) significantly reduced the extent of cicaprost-induced hIP internalization, as evidenced by the strong immunofluorescent staining on the plasma membrane (Figure 3Aiii).

Thereafter, the effect of SCH66336 (1 μM for 24 h) on cicaprost-induced IP internalization was quantified further using an ELISA-based internalization assay [12]. Consistent with previous reports [12], stimulation of hIP with cicaprost induced a time-dependent internalization of the receptor, as monitored by the loss of surface expression of HA-epitope-tagged IP on the plasma membrane in non-permeabilized HEK.hIP cells (Figure 3B; 53.5 ± 1.6 % cell-surface hIP receptor remaining relative to vehicle-treated cells following a 4 h incubation with cicaprost). However, pre-incubation of the HEK.hIP cells with SCH66336

Figure 2 Effect of SCH66336 on IP-mediated $[Ca^{2+}]_i$ mobilization

HEK.hIP cells (A and B), HEK.mIP cells (C and D), HEL cells (E and F) and, as controls, HEK.TP β cells (G and H) were pre-incubated with either 1000 nM SCH66336 or 0.0001 % DMSO (vehicle) (A, C, E and G) or, alternatively, with 0–1000 nM SCH66336 (B, D, F and H) for 16 h before harvesting. Cells, preloaded with fura2/AM, were stimulated with 1 μ M cicaprost (A, C and E) or with 1 μ M U46619 (G) at times indicated by the arrows. Data presented in (A), (C), (E) and (G) are representative of at least four independent experiments, and are plotted as changes (nM) in $[Ca^{2+}]_i \pm$ S.E.M. ($n=4$) as a function of time (s). In panels (B), (D), (F) and (H), mean changes (nM) in $[Ca^{2+}]_i \pm$ S.E.M. ($n=4$) are plotted as a function of SCH66336 concentration (nM).

(1 μ M for 24 h) significantly impaired cicaprost-induced internalization of the hIP (Figure 3B; 82.3 ± 1.6 % cell-surface hIP receptor remaining relative to vehicle-treated cells following 4 h; $P < 0.0001$).

The effect of SCH66336 on IP-mediated desensitization of TP signalling

We have previously established that the TP α , but not the TP β , isoform of TP is subject to IP-induced desensitization/counter-regulation of signalling that occurs through a mechanism involving direct PKA (cAMP-dependent protein kinase) phosphorylation of TP α within its unique C-tail domain, whereby Ser³²⁹ was identified as the target residue for PKA phosphorylation

and receptor desensitization [13]. Thus, in view of its inhibitory effect on IP signalling reported herein, we sought to investigate the effect of SCH66336 on IP-mediated desensitization or counter-regulation of TP receptor responses. Thus, to this end, we examined the ability SCH66336 to regulate cicaprost-induced desensitization of TP α - and, as a control, TP β -mediated increases in $[Ca^{2+}]_i$ mobilization in response to their stimulation with U46616. Consistent with previous reports [13], stimulation of both TP α and TP β , stably overexpressed in HEK.TP α and HEK.TP β cells respectively, showed efficient U46619-induced $[Ca^{2+}]_i$ mobilization (Figures 4A and 4B; $\Delta[Ca^{2+}]_i = 170 \pm 21.8$ nM and $\Delta[Ca^{2+}]_i = 204 \pm 7.9$ nM for TP α and TP β respectively). Whereas stimulation of HEK.TP α cells with cicaprost (1 μ M) failed to induce a rise $[Ca^{2+}]_i$, it significantly reduced $[Ca^{2+}]_i$

Table 2 Radioligand binding assays

(a) Radioligand binding assays were performed on crude cell membrane fractions (P100; 50–100 μ g/assay) in the presence of 4 nM [3 H]iloprost at 30°C for 60 min. (b) Radioligand binding assays were performed on whole cells (50–100 μ g/assay) in the presence of 25 nM [3 H]CGRP-12177 at 14°C for 3 h. (c) Radioligand binding assays were performed on whole cells in the presence of 20 nM [3 H]SQ29,548 at 30°C for 30 min. Data presented are the means $\pm$ S.E.M ($n=3$).

(a)		
Cell type	SCH66336 (1 μ M)	[3 H]iloprost bound (fmol/mg of protein)
HEK.hIP	–	1353 $\pm$ 438
	+	1206 $\pm$ 479
HEK.mIP	–	1362 $\pm$ 72.1
	+	1401 $\pm$ 11.3
HEL	–	87.9 $\pm$ 22.2
	+	95.6 $\pm$ 38.5
HEK-293	–	12.0 $\pm$ 0.59
	+	15.2 $\pm$ 2.23
(b)		
Cell type	SCH66336 (1 μ M)	[3 H]CGRP-12177 bound (fmol/mg of protein)
HEK. β_2 AR	–	329 $\pm$ 28.3
	+	341 $\pm$ 50.1
HEK 293	–	7.82 $\pm$ 0.56
	+	8.65 $\pm$ 0.44
(c)		
Cell type	SCH66336 (1 μ M)	[3 H]SQ29,548 bound (fmol/mg of protein)
HEK.TP α	–	1946 $\pm$ 48
	+	2070 $\pm$ 134
HEK.TP β	–	2158 $\pm$ 126
	+	1716 $\pm$ 93.9
HEK-293	–	12.8 $\pm$ 0.30
	+	14.5 $\pm$ 3.57

mobilization in response to secondary stimulation of cells with U46619 (Figure 4A, compare values for U46619-mediated [Ca^{2+}]_i mobilization: $\Delta[\text{Ca}^{2+}]_i = 170 \pm 21.8$ nM compared with $\Delta[\text{Ca}^{2+}]_i = 57.7 \pm 13.9$ nM in the absence and presence of cicaprost respectively; $P = 0.0124$). Pre-incubation of HEK.TP α cells with SCH66336 (1 μ M) had no significant effect on U46619-induced [Ca^{2+}]_i mobilization {Figure 4C, compare values for U46619-mediated [Ca^{2+}]_i mobilization: $\Delta[\text{Ca}^{2+}]_i = 170 \pm 21.8$ nM (Figure 4A) compared with $\Delta[\text{Ca}^{2+}]_i = 154 \pm 5.20$ nM (Figure 4C) in the absence and presence of SCH66336 respectively; $P = 0.3044$ }. Conversely, pre-incubation of HEK.TP α cells with SCH66336 significantly reduced the level of cicaprost-induced desensitization of TP α -mediated [Ca^{2+}]_i mobilization in response to U46619 {Figure 4C, compare values for U46619-mediated [Ca^{2+}]_i mobilization following pre-stimulation with cicaprost: $\Delta[\text{Ca}^{2+}]_i = 57.7 \pm 13.9$ nM (Figure 4A) compared with $\Delta[\text{Ca}^{2+}]_i = 155 \pm 20.7$ nM (Figure 4C) in the absence and presence of SCH66336 respectively; $P = 0.6565$ }. From concentration–response studies, the IC₅₀ value for inhibition of cicaprost-induced desensitization of TP α signalling was 56.1 nM SCH66336 (Figure 4E). Consistent with previous results [13], stimulation of HEK.TP β cells with U46619 yielded substantial rises in [Ca^{2+}]_i mobilization (Figures 4B and 4D). Stimulation of HEK.TP β cells with cicaprost (1 μ M) failed to support a rise in cicaprost-induced [Ca^{2+}]_i mobilization (Figures 4B and 4D), and also failed to affect the level of TP β -mediated [Ca^{2+}]_i mobilization in response to secondary stimulation of HEK.TP β cells with U46619 (Figure 4B, compare values for U46619-mediated [Ca^{2+}]_i mobilization following pre-stimulation with cicaprost: $\Delta[\text{Ca}^{2+}]_i = 204 \pm 7.9$ nM compared with $\Delta[\text{Ca}^{2+}]_i = 210 \pm 14.8$ nM; $P = 0.7511$). Moreover, pre-incubation of HEK.TP β cells with SCH66336 had no effect on the U46619-induced [Ca^{2+}]_i mobilization (Figure 4D), and had no effect on the ability of cicaprost to affect TP β -mediated [Ca^{2+}]_i mobilization (Figures 4D and 4F). Thus U46619-induced signalling by

Figure 3 Effect of SCH66336 on cicaprost-mediated IP internalization

(A) HEK.hIP cells (A, i–iii) and HEK-293 cells (A, iv) were incubated with 1 μ M SCH66336 (A, iii) or with 0.0001% DMSO (A, i–ii) for 24 h before stimulation with cicaprost for 4 h (1 μ M; A, ii–iv). Thereafter, cells were subject to indirect immunofluorescence microscopy under non-permeabilizing conditions using the anti-HA 101R primary antibody (1:2000 dilution) and FITC-labelled goat anti-mouse IgG (1:120 dilution) secondary antibody. Images captured with a FITC filter using an Axioplan 2 Fluorescent Microscope (Zeiss) represent 63 $\times$ magnification, and are representative of three independent experiments. (B) HEK.hIP cells were pre-incubated for 24 h with 1 μ M SCH66336 (hIP + SCH) or with 0.0001% DMSO (hIP). Thereafter, cells were stimulated with cicaprost (1 μ M) for 0–4 h and internalization of HA-tagged receptors was quantified by ELISA assay, as described in the Experimental section. Results are presented as the mean percentage of cell-surface receptor present in cicaprost-treated cells relative to that present in the corresponding vehicle (MEM)-treated cells $\pm$ S.E.M. (% cell-surface receptor $\pm$ S.E.M.), where vehicle-treated control cells were assigned a value of 100%.

Figure 4 Effect of SCH66336 on IP-mediated desensitization of TP-mediated signalling

HEK.TP α cells (**A**, **C** and **E**) and HEK.TP β cells (**B**, **D** and **F**), transiently transfected with pCMV:G α_q , were pre-incubated with either the vehicle 0.0001% DMSO (**A** and **B**), with 1000 nM SCH66336 (**C** and **D**) or with 0–1000 nM SCH66336 (**E** and **F**) for 16 h before harvesting. Cells, preloaded with fura2/AM, were stimulated with 1 μ M U46619 at 50 s (U46619) or with 1 μ M cicaprost at 150 s followed by 1 μ M U46619 at approx. 150–200 s (Cicaprost, U46619). Data shown in (**A**–**D**) are each representative of four independent experiments, and are plotted as changes (nM) in $\Delta[\text{Ca}^{2+}]_i$ mobilization ($\Delta[\text{Ca}^{2+}]_i \pm \text{S.E.M.}$, $n = 4$) as a function of time (s). In (**E**) and (**F**), data are plotted as mean changes (nM) in $[\text{Ca}^{2+}]_i$ mobilization ($\Delta[\text{Ca}^{2+}]_i \pm \text{S.E.M.}$, $n = 4$) as a function of SCH66336 concentration (nM).

HEK.TP β cells was unaffected, irrespective of pre-incubation of cells in the absence or presence of SCH66336 (Figures 4D and 4F).

The effect of SCH66336 on HDJ-2 isoprenylation

The molecular chaperone protein HDJ-2 undergoes farnesylation-dependent processing, and displays a undergoes shift between the non-farnesylated (49 kDa) and farnesylated (45 kDa) species respectively, such as that displayed when farnesylation is impaired in the presence of FTIs [18]. Thus detection and measurement of the relative abundance of the farnesylated and non-farnesylated forms of HDJ-2 through immunoblot analysis is widely reported to represent a convenient method to (a) assess the potencies of FTI, and (b) in the present study, should allow for a comparison between the relative inhibitory effects of SCH66336 on the isoprenylation of the IP to that of the HDJ-2. Analysis of whole-cell lysates prepared from HEK-293 cells indicated the abundant expression of HDJ-2 protein in that cell line and that, in the absence of the FTI, it is almost exclusively found in its fully processed, isoprenylated form, with no detectable expression of the slower migrating non-farnesylated species (Figure 5A). Following 24 h incubation of HEK-293 cells with SCH66336 (0–

10 nM), there was a concentration-dependent increase in the accumulation of non-farnesylated species, and a co-ordinated reduction in the level of the faster migrating, farnesylated species (Figures 5A and 5C). An IC_{50} value for inhibition of HDJ-2 farnesylation was 3.1 nM SCH66336, whereas the minimum inhibitory concentration for the appearance of the slower migrating non-farnesylated form was detected at 0.8 nM (Figures 5A and 5C). To determine the pre-incubation time required for SCH66336 to inhibit HDJ-2 farnesylation, a time course assay was performed whereby HEK-293 cells were incubated in the presence of SCH66336 (5 nM) for 0–24 h (Figures 5B and 5D). Data generated showed that SCH66336 significantly affected the mobility of HDJ-2 after 4 h, and continued to 24 h (Figure 5B).

The effect of SCH66336 on protein isoprenylation *in vitro*

Whereas all four Ras proteins serve as efficient substrates for FTase, readily undergoing farnesylation both *in vitro* and *in vivo* [4], numerous studies have demonstrated that, in the presence of FTIs, Ki-Ras 4A, Ki-Ras 4B and N-Ras, but not Ha-Ras, may become alternatively geranylgeranylated [19–21]. Thus, in order to establish whether farnesylation of the CaaX motif of the IP with the sequence -CSLC is inhibited by SCH66336, and to establish

Figure 5 Effect of SCH66336 on isoprenylation and processing of HDJ-2

(A) HEK-293 cells were pre-incubated for 24 h with vehicle 0.0001 % DMSO (Veh) or with 0–10 nM SCH66336. (B) HEK-293 cells were pre-incubated for 0–24 h with vehicle 0.0001 % DMSO (Veh) or with 5 nM SCH66336. Thereafter, cells were harvested and aliquots (25 µg) of whole-cell protein were resolved by SDS-PAGE/immunoblotting and screened with the anti-HDJ-2 antibody, as described in the Experimental section. The arrows to the right of (A) and (B) indicate the slower migrating non-farnesylated species (≈ 49 kDa) and the faster migrating, farnesylated species of HDJ-2 (≈ 45 – 46 kDa), whereas the position of the 45 kDa molecular-mass marker is indicated to the left. (C) The extent of inhibition of HDJ-2 farnesylation in (A) is plotted as the mean percentage increase of the non-farnesylated species of HDJ-2 detected in the presence of increasing concentrations of SCH66336 relative to that present in vehicle-treated cells (% inhibition $\pm$ S.E.M., $n = 4$). (D) The extent of inhibition of HDJ-2 farnesylation in (B) is plotted as the mean percentage increase of the non-farnesylated species of HDJ-2 detected in the presence of 5 nM SCH66336 at various time points relative to that present in vehicle-treated cells (% inhibition $\pm$ S.E.M., $n = 4$).

whether this motif is subject to alternative geranylgeranylation in the presence of SCH66336, similar to that seen with N-Ras, Ki-Ras 4A and Ki-Ras 4B, we generated a series of chimaeric Ha-Ras mutants in which we directly replaced the -CaaX motif sequence of wild-type Ha-Ras (-CVLS, corresponding to residues 186–189) with that of IP (-CSLC) to generate Ha-Ras^{CSLC}. Moreover, as controls, other chimaeric Ha-Ras mutants were created by directly replacing the -CaaX motif sequence of Ha-Ras^{WT} (-CVLS, corresponding to residues 186–189) with: (i) that of the non-isoprenylated IP control -SSLC sequence, to generate Ha-Ras^{SSLC}; (ii) that of the geranylgeranylated Rac 1 -CVLL sequence, to generate Ha-Ras^{CVLL}; or (iii) that of the alternatively isoprenylated Ki-Ras 4B -CVIM sequence, to generate Ha-Ras^{CVIM}. This experimental approach of replacing the CAAX motif of Ha-Ras with those of other isoprenylated proteins first allows for a direct comparison between the various -CAAX motifs under study as substrates for isoprenylation, either *in vitro* or in whole cells (*in vivo*). Secondly, in the case of the IP, this approach is necessitated by the inability to translate the seven-transmembrane receptor in an *in vitro* system and, thirdly, by the difficulty in expressing the IP at sufficiently high levels in mammalian systems to facilitate its efficient metabolic labelling with [³H]MVA *in vivo* in intact cells which may, in turn, obscure interpretation of data generated in the presence of SCH66336.

Initially, we employed an *in vitro* assay approach to directly compare the effect of SCH66336 on the isoprenylation and farnesylation of Ha-Ras and its variants. To this end, cDNA sequences encoding Ha-Ras^{WT}, Ha-Ras^{CSLC}, Ha-Ras^{SSLC}, Ha-Ras^{CVLL} and Ha-Ras^{CVIM} were transcribed and translated *in vitro*. Based on the incorporation of [³⁵S]methionine, all proteins were synthesized with comparable efficiencies (Figure 6A, lanes 1–5), whereas the level of endogenous protein synthesis in the absence

Figure 6 Effect of SCH66336 on isoprenylation of the -CSLC motif *in vitro*

RNA transcripts coding for Ha-Ras (lane 1), Ha-Ras^{CSLC} (lane 2), Ha-Ras^{SSLC} (lane 3), Ha-Ras^{CVLL} (lane 4) and Ha-Ras^{CVIM} (lane 5) were translated *in vitro* in the presence of [³⁵S]methionine (A), [³H]MVA (B), [³⁵S]methionine plus MVA (C) or [³H]MVA plus 25 nM SCH66336 (D), as described in the Experimental section. Parallel *in vitro* translations carried out in the absence of exogenous added RNA served as controls for [³⁵S]methionine and [³H]MVA reactions (A–D, lanes 6). Aliquots from each translation reaction (8 µl from [³⁵S]methionine reactions and 25 µl from [³H]MVA reactions) were resolved by SDS/PAGE and electroblotted on to PVDF membranes. Thereafter, blots were subjected to fluorography to visualize the radiolabelled proteins. Blots containing ³⁵S-labelled proteins (A and C) were exposed for 16 h, while those containing ³H-labelled proteins (B and D) were exposed for 21 days. The position of a 20.1 kDa molecular-mass marker (kDa) is indicated to the right of (A)–(D).

of exogenous RNA was negligible (Figure 6A, lane 6). Thereafter, to investigate the effect of SCH66336 on the isoprenylation of Ha-Ras^{WT} and the four chimaeric Ha-Ras mutants, parallel *in vitro* translation reactions were allowed to proceed either in the presence of the radiolabelled isoprenoid precursor [³H]MVA, which can be readily converted into the isoprenoid donors FPP and GGPP by the reticulocyte lysate system [14], or in the presence of the isoprenoid donors [³H]FPP or [³H]GGPP. Again, all proteins were synthesized with comparable efficiencies in the presence of MVA on the basis of the incorporation of [³⁵S]methionine (Figure 6C, lanes 1–6). In the presence of [³H]MVA alone, single protein bands of 21 kDa corresponding to isoprenylated Ha-Ras^{WT}, Ha-Ras^{CSLC}, Ha-Ras^{CVLL} and Ha-Ras^{CVIM} were readily detected (Figure 6B, lanes 1, 2, 4 and 5) whereas no [³H]MVA was incorporated into Ha-Ras^{SSLC} (Figure 6B, lane 3) confirming that mutation of the critical Cys¹⁸⁶ to Ser¹⁸⁶ within the CaaX motif of Ha-Ras^{CSLC} abolished isoprenylation. Moreover, in the absence of exogenous RNA, no detectable [³H]MVA was incorporated into endogenous proteins within the reticulocyte lysate system itself (Figure 6B, lane 6). Whereas the inclusion of 25 nM SCH66336 (Figure 6D; or 1 µM, results not shown) during *in vitro* translation reactions had no effect on the ability to translate the Ha-Ras proteins, as assessed by the incorporation of [³⁵S]methionine (results not shown), it abolished [³H]MVA incorporation into Ha-Ras^{WT} and Ha-Ras^{CSLC} (Figure 6D, lanes 1 and 2) while incorporation of [³H]MVA into Ha-Ras^{CVLL}, a known substrate for GGTase 1, was unaffected by SCH66336 (Figure 6D, lane 4). Consistent with the ability of Ki-Ras to undergo alternative geranylgeranylation in the presence of FTIs [19–21], [³H]MVA incorporation into Ha-Ras^{CVIM} was not affected by SCH66336 (Figure 6D, lane 5). Indeed, incorporation of the mevalonate label into Ha-Ras^{CVIM} was not substantially inhibited by an increased concentration of SCH66336 (1 µM; results not shown).

Figure 7 Effect of SCH66336 on incorporation of [^3H]FPP and [^3H]GGPP into Ras translation products

RNA transcripts coding for Ha-Ras (lane 1), Ha-Ras^{CSLC} (lane 2), Ha-Ras^{CVLL} (lane 3), Ha-Ras^{CVIM} (lane 4) were translated *in vitro* in the presence of [^{35}S]methionine (A), [^3H]FPP (B), [^3H]GGPP (C), [^3H]FPP plus 25 nM SCH66336 (D) or [^3H]GGPP plus 25 nM SCH66336 (E) as described in the Experimental section. Parallel *in vitro* translations carried out in the absence of exogenous added RNA served as controls for [^{35}S]methionine, [^3H]FPP and [^3H]GGPP reactions respectively (A–E, lanes 5). Aliquots from each translation reaction (8 μl from [^{35}S]methionine reactions and 25 μl from [^3H]FPP and [^3H]GGPP reactions) were resolved by SDS/PAGE and electroblotted on to PVDF membranes. Thereafter, blots were subjected to fluorography to visualize the radiolabelled proteins. Blots containing ^{35}S -labelled proteins were exposed for 16 h (A), whereas those containing ^3H -labelled proteins were exposed for 21 days (B–E). The position of a 20.1 kDa molecular-mass marker (kDa) is indicated to the right of (A)–(E).

The specificity of SCH66336 inhibition on the modifications of the Ras proteins by either FPP or GGPP was evaluated further in which Ha-Ras^{WT}, Ha-Ras^{CSLC}, Ha-Ras^{CVLL} and Ha-Ras^{CVIM} were translated in the presence of the direct isoprene donors [^3H]FPP or [^3H]GGPP. In agreement with the [^3H]MVA data, Ha-Ras^{WT}, Ha-Ras^{CSLC} and Ha-Ras^{CVIM} were all efficiently farnesylated (Figure 7A, lanes 1, 2 and 4), whereas the GGTase 1 control substrate Ha-Ras^{CVLL} failed to incorporate any [^3H]FPP label (Figure 7B, lane 3). Inclusion of SCH66336 (25 nM) during the *in vitro* translation reactions inhibited [^3H]FPP incorporation into Ha-Ras^{WT}, Ha-Ras^{CSLC} and Ha-Ras^{CVIM} (Figure 7D, lanes 1, 2 and 4). On the other hand, while Ha-Ras^{WT} and Ha-Ras^{CSLC} failed to incorporate the [^3H]GGPP label (Figure 7C, lanes 1 and 2), both Ha-Ras^{CVLL} and Ha-Ras^{CVIM} were efficiently geranylgeranylated in the presence of [^3H]GGPP (Figure 7C, lanes 3 and 4). Moreover, inclusion of SCH66336 (25 nM) during the *in vitro* translation reaction fully supported the alternative geranylgeranylation of Ha-Ras^{CVIM} (Figure 7E, lane 4). As expected, SCH66336 had no effect on labelling of the GGTase 1 substrate control Ha-Ras^{CVLL} (Figure 7E, lane 3).

The effect of SCH66336 on protein isoprenylation *in vivo*

Thereafter, to investigate whether SCH66336 impairs isoprenylation of the -CSLC motif of the IP *in vivo*, whole-cell metabolic labelling studies were carried out in the presence of [^3H]MVA in HEK-293 cells transiently transfected with plasmids encoding Ha-Ras^{WT}, Ha-Ras^{CSLC}, Ha-Ras^{SSLC}, Ha-Ras^{CVLL} and Ha-Ras^{CVIM}, where cells transfected with the empty vector served as a control. Following metabolic labelling, whole-cell protein was either analysed directly, or HA-epitope-tagged Ras proteins were immunoprecipitated and analysed for labelling by fluorography. In

Figure 8 Effect of SCH66336 on the isoprenylation of the -CSLC motif in whole cells

HEK-293 cells were transiently co-transfected with pMEV along with pHM:Ras^{WT}, pHM:Ras^{CSLC}, pHM:Ras^{SSLC}, pHM:Ras^{CVLL}, pHM:Ras^{CVIM} or, as a negative control, the empty vector pHM6 before metabolic labelling with [^3H]MVA either in the absence (A and C) or the presence (B and D) of SCH66336. (A) and (B) To analyse the effect of SCH66336 on total metabolic labelling, aliquots (60 μg) of whole-cell proteins isolated from cells labelled in the absence (A) or presence (B) of 1 μM SCH66336 were resolved directly by SDS/PAGE, followed by electroblotting on to PVDF membranes, where proteins isolated from the mock (pHM6)-transfected HEK-293 cells are shown as representatives in (A) and (B). (C) and (D) Alternatively, cell lysates from the respective transfected cells (containing approx. 500 μg of protein/lysate) were subjected to immunoprecipitation with anti-HA 101r serum to immunoprecipitate the HA-tagged Ha-Ras^{WT} (lane 1), Ha-Ras^{CSLC} (lane 2), Ha-Ras^{SSLC} (lane 3), Ha-Ras^{CVLL} (lane 4) and Ha-Ras^{CVIM} (lane 5), where cell lysates from mock-transfected HEK-293 cells (pHM6, lane 6) served as negative controls. Immunoprecipitates were resolved by SDS/PAGE, followed by electroblotting on to a PVDF membrane. Blots in (A)–(D) were then soaked in Amplify for 30 min and exposed to X-Omat AR 5 film for 21 days at -70°C . (E) In parallel experiments, HA-tagged proteins immunoprecipitated from non-metabolically labelled HEK-293 cells transiently co-transfected with pMEV, along with either pHM:Ras^{WT} (lane 1), pHM:Ras^{CSLC} (lane 2), pHM:Ras^{SSLC} (lane 3), pHM:Ras^{CVLL} (lane 4), pHM:Ras^{CVIM} (lane 5) or, as a negative control, pHM6 (lane 6) were resolved by SDS/PAGE, followed by electroblotting on to a PVDF membrane; thereafter, membranes were screened using the peroxidase-conjugated anti-HA 3F10 serum followed by chemiluminescent detection. The positions of the molecular-mass markers (kDa) are indicated either to the left or right of (A)–(E), and the position of the isoprenylated 20.1 kDa protein corresponding to Ha-Ras, Ha-Ras^{CSLC}, Ha-Ras^{SSLC}, Ha-Ras^{CVLL} or Ha-Ras^{CVIM} are indicated by an arrow (C–E).

parallel experiments, the quantitative immunoprecipitation and the identities of the precipitated/isoprenylated proteins to be those of the HA-tagged Ras^{WT}, Ras^{CSLC}, Ras^{SSLC}, Ras^{CVLL} and Ras^{CVIM} were each confirmed by direct screening of Western blots with peroxidase-conjugated anti-HA 3F10 antibody (Figure 8E). In the absence of SCH66336, there was efficient uniform isoprenylation as evidenced by the incorporation of [^3H]MVA-derived isoprene units into a host of cell proteins, regardless of transfection type (Figure 8A). Moreover, following immunoprecipitation with the anti-HA antibody, it was evident that Ha-Ras^{WT}, Ha-Ras^{CSLC}, Ha-Ras^{CVLL} and Ha-Ras^{CVIM} were efficiently isoprenylated (Figure 8C, lanes 1, 2, 4 and 5), whereas no [^3H]MVA was incorporated into Ha-Ras^{SSLC} and no [^3H]MVA was immunoprecipitated in the control/mock-transfected HEK-293 cells (Figure 8C, lanes 3 and 6). Inclusion of SCH66336 (1 μM ; or 25 nM, results not shown) during the labelling reaction significantly reduced the incorporation of [^3H]MVA into the range of endogenous cellular isoprenylated proteins, particularly those greater than 45 kDa (Figure 8B) and, more specifically, completely obliterated isoprenylation of Ha-Ras^{WT} and Ha-Ras^{CSLC} (Figure 8D, lanes 1 and 2). Consistent with the *in vitro* data, SCH66336 did not affect isoprenylation of the GGTase 1 substrate Ha-Ras^{CVLL} (Figure 8D, lane 4) or Ha-Ras^{CVIM} (Figure 8D, lane 5), confirming that Ha-Ras^{CVIM} becomes alternatively processed in the presence of strong FTase inhibition.

DISCUSSION

FTIs were originally developed to block constitutive signalling by mutant Ras proteins [22]. However, current evidence suggests that FTIs have broad anticancer action, an activity that in some instances is not necessarily correlated with the presence of mutated *ras* oncogenes [22–24]. Identification of the critical targets or other potential effects of FTI action is the key outstanding question in gaining full knowledge as to the true efficacy of these drugs *in vivo*. SCH66336 is an orally active tricyclic compound that has entered phase II/III clinical trials for the treatment of various cancers, including pancreatic cancer, glioblastoma and solid tumours [5,6]. In addition to its FTase inhibition of Ras, data herein demonstrate that SCH66336 exhibits substantial inhibition of IP function through impairment of IP farnesylation and intracellular signalling.

Along with nitric oxide (NO), prostacyclin acts synergistically on smooth-muscle cells, leucocytes and platelets, playing a major role in maintaining vascular integrity and blood fluidity [7]. Moreover, prostacyclin is a potent pro-inflammatory mediator and has also been proposed as an anticancer prostanoid, given its anti-proliferative, anti-apoptotic and anti-metastatic nature/properties seen in experimental tumour models [25–28]. Although the role of prostacyclin in angiogenesis has not been fully examined, there is evidence for both pro- and anti-angiogenic effects [29–33]. The IP is primarily coupled to G_s -dependent activation of adenylate cyclase, but can also couple to G_q /PLC β [34]. The IP appears to be unique among the GPCRs in that it undergoes farnesylation and palmitoylation, post-translational modifications that are critical for IP function [11,12,15,35].

Given the ambiguity surrounding the mechanism of action of the FTIs and their true targets [5,23,24], the current study examined the effect of SCH66336 on signalling by both the hIP and mIP stably expressed in HEK-293 cells, and by the hIP endogenously expressed in HEL cells. Pre-treatment of HEK.hIP, HEK.mIP and HEL cells with SCH66336 (1 μ M) for 16 h reduced hIP- and mIP-mediated cAMP generation to approx. $28.6 \pm 7.1\%$, $25.7 \pm 9.4\%$ and $19.1 \pm 1.2\%$ respectively, whereas 1 μ M SCH66336 reduced hIP-, mIP- and HEL-mediated $[Ca^{2+}]_i$ mobilization to $37.1 \pm 3\%$, $38.0 \pm 5.5\%$ and $26.8 \pm 7.1\%$ respectively. Moreover, these reductions in IP-mediated cAMP generation and $[Ca^{2+}]_i$ mobilization occurred in a concentration-dependent manner. Conversely, SCH66336 had no effect on $[^3H]$ -iloprost binding, excluding the possibility that the reduction in IP function was attributable to an overall decrease in IP expression. No significant decreases in β_2 AR-mediated cAMP generation or TP β -mediated $[Ca^{2+}]_i$ mobilization occurred, confirming that the reductions elicited by SCH66336 are targeted to the IP itself and not to another component of the signalling system. Whereas SCH66336 (1 μ M) also significantly reduced the extent and kinetics of cicaprost-induced IP internalization, from concentration–response studies it was shown that SCH66336 at concentrations lower than 0.1 μ M did not yield significant reductions in IP internalization (results not shown). Hence, comparing our data herein, it is evident that IP-mediated intracellular signalling (cAMP generation and $[Ca^{2+}]_i$ mobilization) are substantially more sensitive to inhibition by SCH66336 than is agonist-mediated IP internalization. It is, however, indeed noteworthy that the circulating plasma concentrations in humans taking 200 mg of SCH66336 twice daily achieve levels greater than 1 μ M [36], and therefore represent levels substantially higher than those required to inhibit IP signalling and agonist-induced IP internalization in whole cells.

Cross-desensitization or counter-regulation of responses is widely documented to occur between the anti-aggregatory adeny-

late cyclase/PKA system elicited by prostacyclin and the pro-aggregatory PLC β /PKC system elicited by TXA $_2$ in platelets and vascular smooth muscle [8,13,37]. More specifically, the TP α , but not the TP β , isoform of the human TXA $_2$ receptor undergoes IP-mediated desensitization through direct PKA phosphorylation of TP α within its C-tail domain [13]. Given the central role of the latter counter-regulation between IP and TP α signalling, coupled with the finding that SCH66336 is a potent inhibitor of IP function, we next examined the effect of SCH66336 on IP-mediated desensitization or counter-regulation of the TP responses. Consistent with previous studies, both TP α and TP β exhibited efficient U46619-induced $[Ca^{2+}]_i$ mobilization [13,15], a response not significantly altered by SCH66336. Pre-stimulation of the endogenous IP with cicaprost significantly desensitized subsequent U46619-mediated $[Ca^{2+}]_i$ mobilization by TP α , but not by TP β . However, SCH66336 significantly reduced the level of cicaprost-mediated desensitization of TP α signalling in a concentration-dependent manner (IC_{50} 56.1 nM SCH66336). On the other hand, U46619-induced signalling by TP β was unaffected by cicaprost, irrespective of the presence/absence of SCH66336.

The ubiquitously expressed HDJ-2 is farnesylated and undergoes a mobility shift upon treatment with FTI [18]. Thus the detection and relative abundance of the farnesylated and non-farnesylated forms of HDJ-2 through immunoblot analysis offers a convenient method to assess SCH66336 potency and, herein, allowed us to compare the relative inhibitory effects of SCH66336 on the isoprenylation of the IP to that of HDJ-2. Given that the IC_{50} for inhibition of cicaprost-induced cAMP generation reported herein ranged from 0.27 to 0.62 nM SCH66336, and the IC_{50} for inhibition of HDJ-2 isoprenylation was 3.07 nM, it is indeed apparent that IP isoprenylation and primary signalling (agonist-induced cAMP generation) is 5–10-fold more sensitive to SCH66336 inhibition than is HDJ-2.

FTIs offer an appealing paradigm for the rational design and development of novel anticancer agents, such as SCH66336, as they exploit the necessary farnesylation of Ras, and so inhibit its oncogenic potential [5]. However, while all four Ras proteins are efficient substrates for FTase [4], numerous studies have demonstrated that, in the presence of FTIs, N-Ras, Ki-Ras 4A and Ki-Ras 4B may become alternatively geranylgeranylated [19–21,24]. Nonetheless, FTI treatment can significantly inhibit the growth of tumours containing Ki-Ras mutations [38–40], suggesting either that other farnesylated cellular protein(s) distinct from Ras may also play a role in the biological consequences of FTI treatment [41], or that farnesylated and geranylgeranylated Ki-Ras may function differently [24]. Other farnesylated proteins that have been suggested to be possible FTI targets include RhoB, the centromere-binding proteins CENP-E and CENP-F, and the nuclear lamins [23]. However, to date, no protein has been identified that can directly account for all the actions of FTI, and hence studies aimed at investigating their effects on other farnesylated proteins, such as the IP, are critical for the identification of the major targets of FTI and, secondly, for our detailed understanding of the complete mode of action of FTIs.

In the present study, we sought to establish whether SCH66336 could inhibit IP isoprenylation both *in vitro* and *in vivo* and/or whether, analogous to that of Ki-Ras and N-Ras, the IP could become alternatively isoprenylated in the presence of SCH66336. To this end, we generated a series of chimaeric Ha-Ras variants. The experimental approach adopted not only allowed for a direct comparison between the various -CAAX motifs under study, but it was also largely necessitated both by an inability to translate the full-length IP *in vitro* and the relatively inefficient metabolic labelling of the IP with the isoprene precursor $[^3H]$ MVA *in vivo*, thereby potentially obscuring interpretation of data in the presence

of the FTI. In the absence of SCH66336, Ha-Ras^{WT}, Ha-Ras^{CSLC} and Ha-Ras^{CVIM} chimaeras were all preferentially modified by 15-carbon farnesyl isoprenoids both *in vitro* and *in vivo*. Consistent with previous studies [14], changing the terminal serine residue of Ha-Ras to leucine rendered Ha-Ras^{CVLL} a substrate for geranylgeranyl modification *in vitro* and *in vivo*, while mutation of the critical acceptor cysteine of Ha-Ras^{CSLC} to Ha-Ras^{SSLC} completely abolished isoprenylation. In the presence of SCH66336, Ha-Ras^{WT} and Ha-Ras^{CSLC} isoprenylation was completely inhibited, suggesting that, unlike Ki-Ras and N-Ras, the C-terminal regions of both Ha-Ras (-CVLS) and IP (-CSLC) do not undergo alternative geranylgeranylation either *in vitro* or *in vivo*. As expected, inclusion of SCH66336 had no effect on isoprenylation of the control Ha-Ras^{SSLC} or on the geranylgeranylated Rac 1, Ha-Ras^{CVLL} sequence either *in vitro* or *in vivo*. However, SCH66336 resulted in a marked increase in isoprenylation of Ha-Ras^{CVIM}, confirming that, although SCH66336 inhibits farnesylation of the variant Ha-Ras^{CVIM}, it can become alternatively geranylgeranylated in the presence of FTI, and thus serves as a GGase 1 substrate *in vitro* and *in vivo*.

Hence, taken together, these data confirm that the -CSLC motif corresponding to the -CaaX motif of the IP supports the efficient isoprenylation of Ha-Ras and that, unlike the -CVIM motif (naturally found associated with Ki-Ras4B), farnesylation of the -CSLC motif is completely inhibited by SCH66336, and does not undergo alternative geranylgeranylation in the presence of this FTI. Hence, in view of the recognized functional requirement for isoprenylation by the IPs from a range of species [11,12,15], coupled with the observed effects of SCH66336 on IP signalling and function reported herein, it is likely that the IP is a direct target of SCH66336 inhibition which may, in turn, contribute to the more diverse actions of FTIs other than Ras targeting. Whether the therapeutic use of SCH66336 can impair IP-mediated cellular processes *in vivo* or in human subjects is of course currently unknown. However, it is indeed noteworthy that the IC₅₀ values for cAMP generation (IC₅₀ 0.27–0.62 nM) and [Ca²⁺]_i mobilization (IC₅₀ 26.6–48.3 nM) are significantly lower than typical plasma concentrations that are reported to be greater than 1 µM in patients receiving 200 mg of SCH66336 twice daily [36]. In view of this and the data presented herein, the effect of SCH66336 on IP-signalling and function in human subjects remains to be critically addressed at the detailed molecular level.

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REFERENCES

- Adjei, A. A. (2001) Blocking oncogenic Ras signaling for cancer therapy. *J. Natl. Cancer Inst.* **93**, 1062–1074
- Downward, J. (2003) Targeting RAS signalling pathways in cancer therapy. *Nat. Rev. Cancer* **3**, 11–22
- McCormick, F. (1999) Signalling networks that cause cancer. *Trends Cell. Biol.* **9**, M53–M56
- Zhang, F. L. and Casey, P. J. (1996) Protein prenylation: molecular mechanisms and functional consequences. *Annu. Rev. Biochem.* **65**, 241–269
- Caponigro, F. (2002) Farnesyl transferase inhibitors: a major breakthrough in anticancer therapy? Naples, 12 April 2002. *Anticancer Drugs* **13**, 891–897
- Haluska, P., Dy, G. K. and Adjei, A. A. (2002) Farnesyl transferase inhibitors as anticancer agents. *Eur. J. Cancer* **38**, 1685–1700
- Narumiya, S., Sugimoto, Y. and Ushikubi, F. (1999) Prostanoid receptors: structures, properties and functions. *Physiol. Rev.* **79**, 1193–1226
- Cheng, Y., Austin, S. C., Rocca, B., Koller, B. H., Coffman, T. M., Grosser, T., Lawson, J. A. and Fitzgerald, G. A. (2002) Role of prostacyclin in the cardiovascular response to thromboxane A₂. *Science* **296**, 539–541
- Fitzgerald, D. J., Entman, S. S., Mulloy, K. and Fitzgerald, G. A. (1987) Decreased prostacyclin biosynthesis preceding the clinical manifestation of pregnancy-induced hypertension. *Circulation* **75**, 956–963
- Kahn, N. N., Mueller, H. S. and Sinha, A. K. (1990) Impaired prostaglandin E_{1/2} receptor activity of human blood platelets in acute ischemic heart disease. *Circ. Res.* **66**, 932–940
- Hayes, J. S., Lawler, O. A., Walsh, M. T. and Kinsella, B. T. (1999) The prostacyclin receptor is isoprenylated. Isoprenylation is required for efficient receptor-effector coupling. *J. Biol. Chem.* **274**, 23707–23718
- Miggin, S. M., Lawler, O. A. and Kinsella, B. T. (2002) Investigation of a functional requirement for isoprenylation by the human prostacyclin receptor. *Eur. J. Biochem.* **269**, 1714–1725
- Walsh, M. T., Foley, J. F. and Kinsella, B. T. (2000) The alpha, but not the beta, isoform of the human thromboxane A₂ receptor is a target for prostacyclin-mediated desensitization. *J. Biol. Chem.* **275**, 20412–20423
- Kinsella, B. T., Erdman, R. A. and Maltese, W. A. (1991) Posttranslational modification of Ha-ras p21 by farnesyl versus geranylgeranyl isoprenoids is determined by the COOH-terminal amino acid. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8934–8938
- Lawler, O. A., Miggin, S. M. and Kinsella, B. T. (2001) The effects of the statins lovastatin and cerivastatin on signalling by the prostanoid IP-receptor. *Br. J. Pharmacol.* **132**, 1639–1649
- Adjei, A. A., Davis, J. N., Erlichman, C., Svingen, P. A. and Kaufmann, S. H. (2000) Comparison of potential markers of farnesyltransferase inhibition. *Clin. Cancer Res.* **6**, 2318–2325
- Smyth, E. M., Austin, S. C., Reilly, M. P. and Fitzgerald, G. A. (2000) Internalization and sequestration of the human prostacyclin receptor. *J. Biol. Chem.* **275**, 32037–32045
- Kanazawa, M., Terada, K., Kato, S. and Mori, M. (1997) HSDJ, a human homolog of DnaJ, is farnesylated and is involved in protein import into mitochondria. *J. Biochem. (Tokyo)* **121**, 890–895
- Zhang, F. L., Kirschmeier, P., Carr, D., James, L., Bond, R. W., Wang, L., Patton, R., Windsor, W. T., Syto, R., Zhang, R. and Bishop, W. R. (1997) Characterization of Ha-ras, N-ras, Ki-Ras4A, and Ki-Ras4B as *in vitro* substrates for farnesyl protein transferase and geranylgeranyl protein transferase type I. *J. Biol. Chem.* **272**, 10232–10239
- Whyte, D. B., Kirschmeier, P., Hockenberry, T. N., Nunez-Oliva, I., James, L., Catino, J. J., Bishop, W. R. and Pai, J. K. (1997) K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. *J. Biol. Chem.* **272**, 14459–14464
- James, G. L., Goldstein, J. L. and Brown, M. S. (1995) Polylysine and CVIM sequences of K-RasB dictate specificity of prenylation and confer resistance to benzodiazepine peptidomimetic *in vitro*. *J. Biol. Chem.* **270**, 6221–6226
- Gibbs, J. B. and Oliff, A. (1997) The potential of farnesyltransferase inhibitors as cancer chemotherapeutics. *Annu. Rev. Pharmacol. Toxicol.* **37**, 143–166
- Bishop, W. R., Kirschmeier, P. and Baum, C. (2003) Farnesyl transferase inhibitors: mechanism of action, translational studies and clinical evaluation. *Cancer Biol. Ther.* **2**, S96–S104
- Fiordalisi, J. J., Johnson, II, R. L., Weinbaum, C. A., Sakabe, K., Chen, Z., Casey, P. J. and Cox, A. D. (2003) High affinity for farnesyltransferase and alternative prenylation contribute individually to K-Ras4B resistance to farnesyltransferase inhibitors. *J. Biol. Chem.* **278**, 41718–41727
- Honn, K. V., Ciccone, B. and Skoff, A. (1981) Prostacyclin: a potent antimetastatic agent. *Science* **212**, 1270–1272
- Keith, R. L., Miller, Y. E., Hoshikawa, Y., Moore, M. D., Gesell, T. L., Gao, B., Malkinson, A. M., Golpon, H. A., Nemenoff, R. A. and Geraci, M. W. (2002) Manipulation of pulmonary prostacyclin synthase expression prevents murine lung cancer. *Cancer Res.* **62**, 734–740
- Rigas, B., Goldman, I. S. and Levine, L. (1993) Altered eicosanoid levels in human colon cancer. *J. Lab. Clin. Med.* **122**, 518–523
- Schirmer, M., Lichtner, R. B., Graf, H. and Schneider, M. R. (1997) Efficacy of cicaprost on metastasis in advanced tumor disease. *Adv. Exp. Med. Biol.* **400B**, 751–756
- Hiraoka, K., Koike, H., Yamamoto, S., Tomita, N., Yokoyama, C., Tanabe, T., Aikou, T., Ogihara, T., Kaneda, Y. and Morishita, R. (2003) Enhanced therapeutic angiogenesis by cotransfection of prostacyclin synthase gene or optimization of intramuscular injection of naked plasmid DNA. *Circulation* **108**, 2689–2696
- Jones, M. K., Wang, H., Peskar, B. M., Levin, E., Itani, R. M., Sarfeh, I. J. and Tarnawski, A. S. (1999) Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing. *Nat. Med.* **5**, 1418–1423
- Murphy, J. F. and Fitzgerald, D. J. (2001) Vascular endothelial growth factor induces cyclooxygenase-dependent proliferation of endothelial cells via the VEGF-2 receptor. *FASEB J.* **15**, 1667–1669
- Murphy, J. F., Steele, C., Belton, O. and Fitzgerald, D. J. (2003) Induction of cyclooxygenase-1 and -2 modulates angiogenic responses to engagement of alphavbeta3. *Br. J. Haematol.* **121**, 157–164

- 33 Wharton, J., Davie, N., Upton, P. D., Yacoub, M. H., Polak, J. M. and Morrell, N. W. (2000) Prostacyclin analogues differentially inhibit growth of distal and proximal human pulmonary artery smooth muscle cells. *Circulation* **102**, 3130–3136
- 34 Wise, H. and Jones, R. L. (1996) Focus on prostacyclin and its novel mimetics. *Trends Pharmacol. Sci.* **17**, 17–21
- 35 Miggin, S. M., Lawler, O. A. and Kinsella, B. T. (2003) Palmitoylation of the human prostacyclin receptor. Functional implications of palmitoylation and isoprenylation. *J. Biol. Chem.* **278**, 6947–6958
- 36 Eskens, F. A., Awada, A., Cutler, D. L., de Jonge, M. J., Luyten, G. P., Faber, M. N., Statkevich, P., Sparreboom, A., Verweij, J., Hanauske, A. R. and Piccart, M. (2001) PHase I and pharmacokinetic study of the oral farnesyl transferase inhibitor SCH 66336 given twice daily to patients with advanced solid tumors. *J. Clin. Oncol.* **19**, 1167–1175
- 37 Coleman, R. W., Marder, V. J., Salzman, E. W. and Hirsh, J. (1994) Hemostasis and Thrombosis: Basic Principles and Clinical Practice, Philadelphia, Lippincott
- 38 Sun, J., Qian, Y., Hamilton, A. D. and Sebt, S. M. (1995) Ras CAAX peptidomimetic FTI 276 selectively blocks tumor growth in nude mice of a human lung carcinoma with K-Ras mutation and p53 deletion. *Cancer Res.* **55**, 4243–4247
- 39 Suzuki, N., Urano, J. and Tamanoi, F. (1998) Farnesyltransferase inhibitors induce cytochrome c release and caspase 3 activation preferentially in transformed cells. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 15356–15361
- 40 Suzuki, N., Del Villar, K. and Tamanoi, F. (1998) Farnesyltransferase inhibitors induce dramatic morphological changes of KNRK cells that are blocked by microtubule interfering agents. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 10499–10504
- 41 Tamanoi, F., Kato-Stankiewicz, J., Jiang, C., Machado, I. and Thapar, N. (2001) Farnesylated proteins and cell cycle progression. *J. Cell. Biochem. Suppl.* **37**, 64–70

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