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Investigation of the effect of the farnesyl protein transferase inhibitor R115777 on isoprenylation and intracellular signalling by the prostacyclin receptor

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1 The human (h) and mouse (m) prostacyclin receptors (IPs) undergo isoprenylation through attachment of a C-15 farnesyl moiety within their conserved carboxyl terminal -CSLC sequences.

2 Herein, the effects of a novel farnesyl transferase inhibitor R115777 on signalling by the hIP and mIP, overexpressed in human embryonic kidney 293 cells, and by the hIP endogenously expressed in human erythroleukaemia cells were investigated.

3 R115777 significantly impaired IP-mediated cyclic AMP generation (IC_{50} 0.37–0.60 nM) and intracellular calcium ($[Ca^{2+}]_i$) mobilization (IC_{50} 37–65 nM), but had no effect on signalling by the control nonisoprenylated β_2 adrenergic receptor or the α or β isoforms of the human thromboxane A_2 receptor (TP).

4 Additionally, R115777 significantly reduced IP-mediated cross-desensitization of signalling by the TPα, but not by the TPβ, isoform of the human TP and impaired the farnesylation-dependent processing of the chaperone HDJ-2 protein (IC_{50} 4.5 nM).

5 Furthermore, R115777 fully impaired isoprenylation of both the Ha-Ras^{WT} and Ha-Ras^{CSLC} *in vitro* and in whole cells confirming that, unlike N-Ras and Ki-Ras, the -CSLC motif associated with the IP cannot support alternative geranylgeranylation in the presence of R115777 and does not act as a substrate for geranylgeranyl transferase 1 *in vitro* or in whole cells.

6 In conclusion, these data confirm that R115777 potently impairs IP isoprenylation and signalling, and suggest that clinically it may not only target Ras proteins but may also disrupt IP isoprenylation, events which could impact on physiologic processes in which prostacyclin and its receptor are implicated.

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Abbreviations: $[Ca^{2+}]_i$, intracellular calcium; FBS, foetal bovine serum; FPP, farnesylpyrophosphate; FTase, farnesyl protein transferase; GGPP, geranylgeranylpyrophosphate; GGTase, geranylgeranyl protein transferase; GPCR, G protein-coupled receptor; Ha, Harvey; HA, haemagglutinin; HEK, human embryonic kidney; HEL, human erythroleukaemia; HMG CoA, hydroxy methyl glutaryl coenzyme A; IP, prostanoid-IP receptor; Ki, Kirsten; LDH, lactate dehydrogenase; LMMGP, low molecular mass guanine nucleotide-binding protein; MVA, mevalonate; N, neuronal; PG, prostaglandin; PLC, phospholipase C; PVDF, polyvinylidene difluoride; TP, prostanoid-TP receptor; TXA_2, thromboxane A_2

Introduction

Prostaglandin (PG) I_2, or prostacyclin, acts as a potent inhibitor of platelet aggregation and as a vasodilator, counteracting the actions of thromboxane (TX)A_2, for example (Vane & Botting, 1995). As a member of the G protein-coupled receptor (GPCR) superfamily, the prostacyclin receptor (IP) primarily couples to $G\alpha_s$-dependent activation of adenylyl cyclase but may also to couple to phospholipase (PL) Cβ activation and to mobilization of intracellular calcium ($[Ca^{2+}]_i$) (Namba *et al*., 1994; Wise & Jones, 1996). Alterations or imbalances in prostacyclin, its synthase or its IP receptor have been implicated in a number of cardiovascular disorders, including thrombosis, atherosclerosis, myocardial infarction, as well as many other peripheral vascular diseases (Namba *et al*., 1994; Narumiya *et al*., 1999).

Over the past decade or so, protein isoprenylation has been subject to intense research, owing to the fact that several key cellular proteins undergo this type of post-translational lipid modification (Zhang & Casey, 1996). Protein isoprenylation involves the covalent attachment of either carbon-15 farnesyl or carbon-20 geranylgeranyl isoprenoids to conserved Cys residue(s) within specific C-terminal isoprenylation motifs (Zhang & Casey, 1996). Farnesyl protein transferase (FTase) attaches a farnesyl moiety to proteins that contain a 'CAAX' motif, where C represents the acceptor Cys, A is an aliphatic amino acid and X is any amino acid except leucine or isoleucine (Moores *et al*., 1991; Reiss *et al*., 1991), while geranylgeranyl protein transferase (GGTase) I and II transfer geranylgeranyl unit(s) to proteins containing a -CAAX motif

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in which X is leucine or isoleucine and to proteins terminating in -CC or -CXC, respectively (Seabra *et al*., 1992).

The biological significance of isoprenylation is perhaps best exemplified by studies on the ras oncogene product $p21^{Ras}$. The Ras proto-oncoproteins are synthesized as precursors and undergo a series of sequential post-translational modifications, including isoprenylation, proteolytic 'AAX*ing*', carboxymethylation and palmitoylation to yield the functionally active, membrane-associated protein (Zhang & Casey, 1996). The first of these modifications is isoprenylation and, more specifically, in the cases of p21 Harvey (Ha)-, Kirsten (Ki)- and neuronal (N)-Ras, involves farnesylation (Zhang & Casey, 1996). Inhibition of Ras isoprenylation, using selective inhibitors of isoprenylation or by site-directed mutagenesis, not only blocks membrane association but also completely prevents Ras function, including its cell-transforming capability (Kinsella *et al*., 1991b; Cox *et al*., 1994; Kohl *et al*., 1994; 1995). Mutated forms of the ras oncogene are associated with over 30% of human cancers and therefore have long been considered as valid *anti*-cancer targets (Bos, 1989). Thus, the current development of FTase inhibitors aims to prevent the obligatory farnesylation of $p21^{Ras}$ impairing its membrane association and blocking Ras function (Cox & Der, 1997; End, 1999; Kohl, 1999; Rowinsky *et al*., 1999; Adjei, 2001). One such inhibitor, R115777 (Johnson & Johnson Pharmaceutical Research & Development), has shown much promise, having entered phase II clinical trials as an *anti*-cancer drug in solid and haematological malignancies (Zujewski *et al*., 2000; End *et al*., 2001; Karp *et al*., 2001b; Johnston *et al*., 2003).

Although, as stated, FTase inhibitors were originally conceived as anti-Ras targets, several other proteins involved in key physiological processes undergo farnesylation, as opposed to geranylgeranylation, including lamins A and B, Gγ transducin, rhodopsin kinase, CENP-E and CENP-F and the co-charperone DnaJ/HDJ-2 protein, raising the possibility that FTase inhibitors may not only target Ras proteins but may also interfere with the isoprenylation and function of other vital cell proteins (Clarke, 1992; Cox & Der, 1992; Inglese *et al*., 1992; James *et al*., 1994; Zhang & Casey, 1996; Adjei *et al*., 2000; Ashar *et al*., 2000; Terada & Mori, 2000). The recent identification of a conserved 'CAAX' motif, with the sequence -CSLC, within the C-termini of the prostacyclin receptor (IP) from a number of species, led to the establishment that both the mouse (m) and human (h) IPs are isoprenylated through farnesylation *in vivo* (Hayes *et al*., 1999; Miggin *et al*., 2002). Impairment of isoprenylation of the mIP and hIP established that, while it has no influence on ligand binding, it is absolutely required for efficient IP : effector coupling and hence for IP function (Hayes *et al*., 1999; Lawler *et al*., 2001; Miggin *et al*., 2002). The finding that the IP is among the limited class of isoprenylated proteins that undergo farnesylation implies that the FTase inhibitors may also impair IP isoprenylation and signalling, and that their use in the clinic may not only target Ras proteins but may also impair prostacyclin action, events which could influence processes in which prostacyclin and its receptor IP are implicated. Thus, the aim of this study was to investigate the effect of the aforementioned novel nonpeptide tricyclic FTase inhibitor R115777 on isoprenylation and signalling by both the mIP and hIP and to determine whether the IP is a true target for inhibition by R115777. We demonstrate that R115777 potently impairs signalling by both the hIP and mIP in a time- and concentration-dependent manner, and confirm that the 'CAAX' motif of the IP is uniquely a substrate for farnesylation and that in the presence of R115777 the IP does not undergo alternative geranylgeranylation either *in vitro* or in whole cells.

Methods

Materials

R115777 was obtained from Janssen Pharmaceuticals, Berese, Belgium. Cicaprost was a gift from Schering AG (Berlin, Germany). Iloprost, [^{3}H]iloprost (15.3 Ci mmol^{-1}), [^{3}H]-CGP-12177 (41.0 Ci mmol^{-1}) and polyvinylidene difluoride (PVDF) filters were purchased from Amersham Pharmacia Biotech, Buckinghampshire, U.K. [^{3}H]mevalonolactone (20 Ci mmol^{-1}), [^{3}H]farnesylpyrophosphate (FPP) (20 Ci mmol^{-1}), [^{3}H]geranylgeranylpyrophosphate (GGPP) (15–30 Ci mmol^{-1}), [^{35}S]methionine (1175 Ci mmol^{-1}; 10 mC ml^{-1}) and [^{3}H]cAMP (15–30 Ci mmol^{-1}) were purchased from American Radiolabeled Chemicals Inc. Isoproterenol was purchased from Sigma, MO, U.S.A. Fura 2/AM and U46619 were purchased from Calbiochem, Darmstadt, Germany. Rabbit reticulocyte translation system (minus methionine), T7 RNA polymerase, RNasin and all restriction endonucleases were purchased from Promega Corp., Madison, U.S.A. *Taq* DNA polymerase, the chemiluminescence Western blot detection kit and rat monoclonal 3F10 *anti*-haemagglutinin (HA) peroxidase-conjugated antibody were purchased from Roche, East Sussex, U.K. Horseradish peroxidase-conjugated goat *anti*-mouse IgG was from Santa Cruz Biotechnology, CA, U.S.A. Mouse monoclonal 101R *anti*-HA antibody was obtained from BabCO, Berkeley, U.S.A. *Anti*-HDJ-2 antibody was from Neomarkers, CA, U.S.A. Oligonucleotides were synthesized by Sigma Genosys Biotechnologies, St Louis, MO, U.S.A.

Methods

Cell culture and transfections Human embryonic kidney (HEK).mIP, HEK.hIP, HEK.TPα and HEK.TPβ cells stably overexpressing HA epitope-tagged forms of the wild-type mouse (m), human (h) prostacyclin receptor (IP) and the human thromboxane (TX) A_2 receptor (TP) α and β isoforms, respectively, have been described previously (Hayes *et al*., 1999; Walsh & Kinsella, 2000; Lawler *et al*., 2001). HEK 293 cells were transfected with pADVA (10 μg per 10 cm dish) and pHM- or pCMV-based vectors (25 μg per 10 cm dish) using the calcium phosphate/DNA co-precipitation procedure (Graham & van der Eb, 1973). For transient transfections, cells were harvested 48 h post transfection.

To generate a mammalian cell line overexpressing the β_2 adrenergic receptor (AR), the full-length cDNA for the human β_2AR was subcloned from the plasmid pTF3 (American Type Culture Collection) into a *Hin*dIII–*Eco*RI site of pHM6 (Roche) to generate pHM: β_2AR. HEK.β_2AR cell lines stably overexpressing the β_2AR receptor in HEK 293 cells were established, using pHM:β_2AR, and characterized essentially as described previously (Hayes *et al*., 1999).

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 Harvey (Ha)-Ras (Ha-Ras^{WT}) from pCMV5:Ras (Kinsella *et al*., 1991b) into the *Hin*dIII site of pHM6 (Roche). To generate Ha-Ras^{CSLC}, the carboxyl-terminal CAAX motif of Ha-Ras^{WT}, with the sequence Ha-Ras^{CVLS}, was mutated to that of the IP, with the sequence IP^{CSLC}. Specifically, site-directed mutagenesis of Val^{187} and Ser^{189} of Ha-Ras^{WT} to Ser^{187} and Cys^{189}, respectively, to generate Ha-Ras^{CSLC} was performed using the polymerase chain reaction (PCR) employing pCMV:Ras (Kinsella *et al*., 1991b) as a template and 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, Ha-Ras^{WT}) in combination with the specific mutator oligonucleotide 5′-dCTC AAG CT TCA G***C***A GAG C***GA*** ACA CTT GCA GCT CAT GC-3′ (antisense primer; the sequence complimentary to the mutator bases is highlighted in boldface italics). Similarly, to generate Ha-Ras^{SSLC}, Cys^{186}, Val^{187} and Ser^{189} of Ha-Ras^{WT} were converted to Ser^{186}, Ser^{187} and Cys^{189}, respectively, using the latter sense primer and the specific mutator oligonucleotide 5′-dCTC AAG CT TCA G***C***A GAG C***GA*** A***G***A CTT GCA GCT CAT GCA GC-3′ (antisense primer; the sequence complimentary to the mutator bases is highlighted in boldface italics). To generate Ha-Ras^{CVLL}, Ser^{189} of Ha-Ras^{WT} was converted to Leu^{189} using the latter sense primer and the mutator oligonucleotide 5′-dCTC AAG CT TCA ***GAG*** GAG CAT ACA CTT GCA GC-3′ (antisense primer; the sequence complimentary to the mutator bases is highlighted in boldface italics). Finally, to generate Ha-Ras^{CVIM}, Leu^{188} and Ser ^{189} of Ha-Ras^{WT} were converted to Ile^{188} and Met^{189}, respectively, using the latter sense primer and the mutator oligonucleotide 5′-dAGA GGT ACC TTA ***CAT AAT*** CAC ACA CTT GCA GCT-3′ (antisense primer; the sequence complimentary to the mutator bases is highlighted in boldface italics).

In each case, PCR-generated products were subcloned in-frame into the *Hin*dIII site of pHM6 to generate the plasmids pHM:Ras^{CSLC}, pHM:Ras^{SSLC}, pHM:Ras^{CVLL} or, in the case of Ras^{CVIM}, into *Hin*dIII–*Kpn*I 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 Radioligand-binding assays of the IP were carried out as described previously (Hayes *et al*., 1999). Where specified, cells were pre-incubated with either 1 μM R115777 (added from 1000-fold concentrated stocks diluted in 0.0001% DMSO) or with the corresponding volume of vehicle (DMSO), for 16 h prior to harvesting. IP receptor radioligand-binding assays were carried out on crude cell membranes (P_{100} fractions) in the presence of 4 nM [^{3}H]iloprost (15.3 Ci mmol^{-1}) at 30°C for 1 h using 100 μg of protein in 100 μl reactions essentially as described previously (Hayes *et al*., 1999). β_2AR radioligand-binding assays were carried out on whole cells using 25 nM [^{3}H]CGP-12177 (41 Ci mmol^{-1}) at 14°C for 3 h using 100 μg of Ci mmol protein in 100 μl reactions, essentially as described previously (Gagnon *et al*., 1998). TP radioligand-binding assays were carried out on whole cells using 20 nM [^{3}H]SQ29,548 (39 Ci mmol^{-1}) at 30°C for 30 min using 100 μg of protein in 100 μl reactions essentially as described previously (Kinsella *et al*., 1997).

Measurement of R115777-induced cytotoxicity Possible cytotoxic effects of R115777 were evaluated by measuring lactate dehydrogenase (LDH) release following exposure of cells for 24 h with 1, 20 and 1000 nM R115777 using the Cytotox 96 Non-Radioactive Cytotoxicity assay kit (Promega) essentially as described by the manufacturer. The percent of LDH released from the cells was determined by expressing the level of R115777-induced LDH released as a fractional percentage of the maximum LDH release (% release = LDH activity in R115777-treated cells/maximum LDH activity in cell lysate).

Measurement of cAMP To investigate the effect of R115777 on agonist-mediated cAMP generation, cells were pre-incubated in the presence or absence of R115777 (10 nM) for 16 h prior to harvesting. For concentration–response studies, cells were pre-incubated for 16 h in the presence of R115777 (0–10 nM). For time course assays, cells were pre-incubated for 0–24 h in the presence of R115777 (5 nM). Thereafter, nonviable cells were removed and cAMP assays were preformed as described previously by Hayes *et al*. (1999). HEK.hIP, HEK.mIP and human erythroleukaemia (HEL) cells were stimulated with cicaprost (1 μM) or in the case of the HEK.β_2AR cells with isoproterenol (10 μM). In each case, basal cAMP levels (pmol cAMP mg^{-1} cell protein ± s.e.m.) 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, are expressed and presented as fold stimulation of basal (fold increase in cAMP ± s.e.m., $n = 4$).

Measurement of intracellular [Ca^{2+}] mobilization Measurements of [Ca^{2+}]$_i$ mobilization in Fura2/AM-preloaded cells was carried out essentially as described previously (Kinsella *et al*., 1997). Cells were pre-incubated for 16 h with either R115777 (1000 nM) or with the vehicle, 0.0001% DMSO. For concentration–response studies, cells were pre-incubated with R115777 (0–1000 nM) for 16 h. Thereafter, Fura2/AM-preloaded cells were stimulated with the IP agonist cicaprost (1 μM) or, as a control, with the TP agonist U46619 (1 μM). In separate experiments, to examine the concentration effect of R115777 on IP-mediated counter-regulation of TP-mediated signalling, HEK.TPα cells and HEK.TPβ cells, transiently transfected with pCMV:Gα_q, were pre-incubated in the presence and absence of R115777 (1000 nM) for 16 h. For concentration–response studies, cells were pre-incubated with R115777 (0–1000 nM) for 16 h. Thereafter, Fura2/AM-preloaded cells were stimulated with the IP agonist cicaprost (1 μM), followed by stimulation with the TP agonist U46619 (1 μM). The results, representative of at least four independent experiments, are plotted as changes in [Ca^{2+}]$_i$ mobilization (Δ[Ca^{2+}]$_i$ ± s.e.m. nM, $n = 4$) as a function of time (s).

Effect of R115777 on isoprenylation and processing of HDJ-2 protein HEK 293 cells were seeded at 1.5×10^6 cells per 10 cm dish in MEM, 10% FCS. After 24 h, cells were incubated for a further 24 h in the presence of 0–10 nM R115777 or, as controls, in the presence of an equivalent volume of the vehicle (0.0001% DMSO) or, for time-course studies, in the presence of 5 nM R115777 for 0–24 h. Thereafter, cells were harvested and aliquots of whole-cell protein (25 μg) were resuspended in 1 × solubilization buffer (10%

β-mercaptoethanol (v v^{-1}), 2% SDS (w v^{-1}), 30% glycerol (v v^{-1}), 0.025% bromphenol blue (w v^{-1}), 50 mM Tris–HCl, pH 6.8; 60 μl). Samples were boiled for 10 min and were resolved by SDS–PAGE, using 8% polyacrylamide gels, and electroblotted onto PVDF membranes. Thereafter, blots were screened using the *anti*-HDJ-2 (1 : 4000) antibody, followed by a secondary horseradish peroxidase-conjugated goat *anti*-mouse IgG (1 : 8000) antibody. Proteins were visualized using the chemiluminescence detection system, according to the supplier's instructions (Roche). Quantification of farnesylated and nonfarnesylated HDJ-2 protein fractions was performed by densitometric scanning of these blots using a UVP gel documentation system employing the Gelworks 1D Intermediate software. Percentage inhibition of HDJ-2 farnesylation was determined by comparing the signal in the nonfarnesylated protein band with the overall intensities of farnesylated plus nonfarnesylated protein bands. The levels of nonfarnesylated 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 μg) were transcribed as described previously (Maltese & Sheridan, 1990) with T7 RNA polymerase, according to the supplier's instructions (Promega).

In vitro translation of the RNA transcripts was carried out in a methionine-deficient rabbit reticulocyte lysate system (Promega) according to manufacturer's instructions. Standard reactions (25 μl) containing 2.5 μg of RNA and 20 μCi of L-[^{35}S]methionine were incubated for 1 h at 30°C, followed by 1 h at 37°C. Reactions carried out in the absence of added exogenous RNA served as controls for background incorporation. In parallel experiments, the ability of the *in vitro* translated products to undergo isoprenylation was demonstrated by replacing the radiolabelled L-methionine with an equivalent concentration of unlabelled methionine and by supplementing the IVT reaction with [^{3}H]mevalonolactone (MVA) (25 μCi, 20 Ci mmol^{-1}). In order to directly compare the ability of Ha-Ras^{WT}, pHM:Ras^{CSLC}, pHM:Ras^{SSLC}, pHM:Ras^{CVLL} and pHM:Ras^{CVIM} to serve as substrates for modification by either 15-carbon farnesyl groups or 20-carbon geranylgeranyl groups, translation reactions were carried out without radiolabelled precursors for 1 h at 30°C. Reactions were then optimized for either farnesylation or geranylgeranylation; for farnesylation, 1.25 μCi [^{3}H]FPP (20 Ci mmol^{-1}), 20 mM $MgCl_2$ and 5 mM dithiothreitol (DTT) were added to the reaction, and the incubation was continued for 1 h at 37°C; for geranylgeranylation, 1.25 μCi [^{3}H]GGPP (20 Ci mmol^{-1}), 10 μM GGPP, 20 mM $MgCl_2$ and 5 mM DTT were added to the reaction, and the incubation was continued for 1 h at 37°C. To investigate the ability of R115777 to interfere with isoprenylation of Ha-Ras^{WT}, Ha-Ras^{CSLC}, Ha-Ras^{SSLC}, Ha-Ras^{CVLL} and Ha-Ras^{CVIM}, *in vitro* translation reactions (25 μl) were carried out under identical conditions in the presence of 25 nM R115777. Aliquots of each reaction were subject to SDS–PAGE, followed by electroblotting onto PVDF membrane. Blots were soaked in Amplify (Amersham) for 30 min, followed by fluorography using Kodak Xomat XAR film at −70°C for at least 21 days for [^{3}H]MVA/FPP/GGPP exposures or for 16 h for [^{35}S]methionine exposures.

In vivo *isoprenylation in whole cells* To investigate the ability of R115777 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 (Hayes *et al*., 1999). Briefly, HEK 293 cells were seeded at 4.4×10^6 cells per 10 cm dish in 8 ml MEM, 10% foetal bovine serum (FBS); 24–36 h post seeding, cells were transiently co-transfected with pHM:Ras recombinant plasmids (25 μg per 10 cm dish) plus pMEV (10 μg per 10 cm dish), encoding the membrane-bound mevalonolactone transporter, plus pADVA (10 μg per 10 cm dish). As a negative control, HEK 293 cells were co-transfected with pHM6 (25 μg per 10 cm dish) plus pMEV (10 μg per 10 cm dish) plus pADVA (10 μg per 10 cm dish). At 4 h post transfection, lovastatin (10 μM) was added to the cells to deplete the intracellular pool of mevalonate (MVA) and its metabolites. After 4 h incubation, the medium was replaced with fresh medium (3 ml MEM plus 10% heated inactivated FBS per 10 cm dish) containing either 250 μCi [^{3}H]mevalonolactone (20 Ci mmol^{-1}) plus 1 μM R115777 or 250 μCi [^{3}H]mevalonolactone (20 Ci mmol^{-1}) plus an equivalent volume of the drug vehicle (0.0001% DMSO). Following incubation at 37°C for 16 h, cells were harvested and aliquots of whole-cell protein (60 μg) were resolved by SDS–PAGE and electroblotted onto PVDF membrane. The remaining protein from each transfection ($\sim$500 μg) was subjected to immunoprecipitation using the anti-(HA 101R) serum as described previously (Hayes *et al*., 1999). Immunoprecipitates were resolved by SDS–PAGE and electroblotted onto PVDF membrane. Blots were soaked in Amplify (Amersham) for 30 min, followed by autoradiography using Kodak Xomat XAR film at −70°C for up to 30 days to detect ^{3}H-labelled proteins. Thereafter, membranes were screened by immunoblot analysis using the peroxidase-conjugated anti-(HA 3F10) serum, followed by chemiluminescence detection. To confirm equivalent protein expression of the various Ha-Ras proteins under study and to confirm equal protein loading, in parallel experiments, nonmetabolically labelled transfected cells were subject to identical conditions to [^{3}H]MVA-labelled cells, except that an equivalent volume and concentration of MVA replaced the [^{3}H]MVA. Thereafter, non-labelled HA-tagged Ha-Ras proteins were immunoprecipitated with the anti-(HA 101R) serum and, following SDS–PAGE and Western transfer, immunoblots were screened with the peroxidase conjugated anti-(HA 3F10) serum, followed by chemiluminescence detection.

Data analysis Statistical analysis was carried out using the unpaired Student's *t*-test using GraphPad Prism V2.0 programme (GraphPad Software Inc., San Diego, CA, U.S.A.). *P*-values of less than or equal to 0.05 were considered to indicate a statistically significant difference.

Results

Effect of R115777 on IP receptor-mediated cyclic AMP generation

In the current study, we investigated the effect of the FTase inhibitor R115777 on the signalling and processing of the mIP and hIP either stably overexpressed in HEK 293 cells or on the hIP endogenously expressed in human erythroleukemia (HEL)

92.1.7 cells. Initially, any potential cytotoxic effects of R115777 in either the parental HEK 293 or HEL cell lines was assessed in order to exclude the possibility that any apparent inhibition of IP signalling was not simply due to cytotoxicity *per se* but rather was due to impairment of IP isoprenylation, signalling and function. Concentration–response studies revealed that the level of cytotoxicity in both cell types was low but became significant at 20 nM (5.5 ± 0.2 and $8.2 \pm 0.8\%$ cytotoxicity of HEL and HEK 293 cells, respectively), but that greater than 75% of cells remained viable even at the highest concentration of R115777 (1000 nM) examined. Similar data were generated in the clonal HEK.hIP and HEK.mIP cell lines stably overexpressing the hIP and mIP, respectively (data not shown). Hence, for further studies outlined herein, the effect of R115777 on IP function was performed on viable cells only.

Thereafter, the effect of R115777 on signalling by the hIP and mIP or, serving as a control for a nonisoprenylated Gs/adenylyl cyclase-coupled receptor, by the β_2 adrenergic receptor (AR) was initially determined by examining agonist-mediated cAMP generation in response to the selective IP agonist cicaprost (1 μM) or the β_2AR agonist isoproterenol (10 μM), respectively. While stimulation of both HEK.hIP and HEK.mIP cells each produced substantial increases in cicaprost-mediated cAMP generation, pre-incubation with 10 nM R115777 for 16 h significantly impaired signalling in both cell types (Figure 1a; $P \leqslant 0.0001$). From concentration–response studies, the inhibitory concentration 50 (IC_{50}) values were 0.41 nM R115777 in HEK.hIP cells (Figure 1b) and 0.37 nM R115777 in HEK.mIP cells (Figure 1c), respectively, while the extents of maximal inhibition at 10 nM R115777 were $81 \pm 0.3\%$ ($P \leqslant 0.0001$) and $86.5 \pm 3.7\%$ ($P \leqslant 0.0001$) for the hIP and the mIP, respectively (Figure 1a and c). In HEL cells, stimulation of the hIP endogenously expressed in this megakaryocytic cell line with cicaprost yielded substantial increases in cAMP generation (Figure 1a and d); however, pre-incubation of HEL cells with R115777 (16 h) reduced its signalling in a concentration-dependent manner, with an IC_{50} of 0.6 nM R115777 (Figure 1d), while the extent of maximal inhibition of signalling in these cells at 10 nM R115777 was $82.5 \pm 2.8\%$ (Figure 1a and d; $P \leqslant 0.0001$). In contrast, while stimulation of HEK.β_2AR cells with isoproterenol yielded significant increases in cAMP generation, pre-incubation of those cells with R115777 had no significant effect on signalling by the β_2AR even at the highest R115777 concentrations employed (Figure 1a and e, $P \geqslant 0.05$). In time-course assays, R115777 (5 nM) significantly reduced cicaprost-induced cAMP generation in HEK.hIP, HEK.mIP and HEL cells following

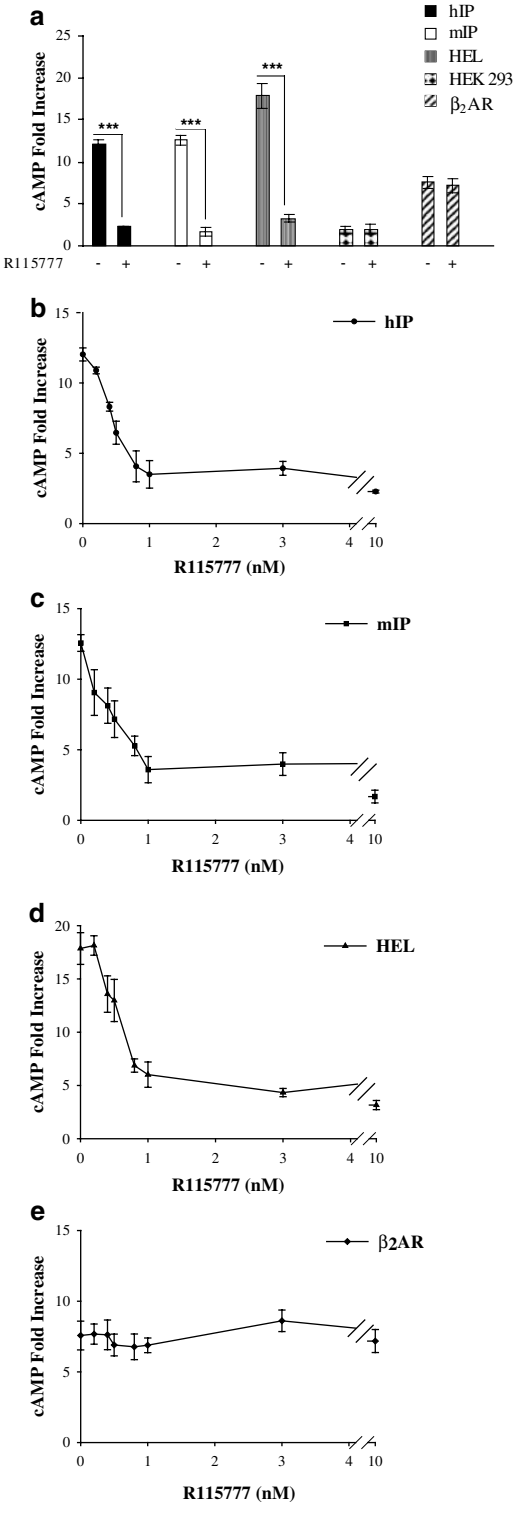

Figure 1 Effect of R115777 on IP-mediated cAMP generation. HEK.hIP cells (hIP), HEK.mIP cells (mIP), HEL cells (HEL), HEK 293 cells or, as controls, HEK.β_2AR cells (β_2AR) were pre-incubated with (+) or without (−) 10 nM R115777 for 16 h prior to harvesting (panel a). HEK.hIP cells (hIP: panel b), HEK.mIP cells (mIP: panel c), HEL cells (HEL: panel d), or HEK.β_2AR cells (β_2AR: panel e) were pre-incubated with 0–10 nM R115777 for 16 h prior to harvesting. Thereafter, cells were stimulated with 1 μM cicaprost or, in the case of the HEK.β_2AR 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 (*) indicate that the level of cicaprost-mediated cAMP generation was significantly reduced in the presence of R115777 compared to vehicle-treated cells, where *** indicates $P \leqslant 0.0001$. Basal levels of cAMP in HEK.hIP, HEK.mIP, HEL, HEK 293 and in HEK.β_2AR cells were 0.51 ± 0.07 nmol mg^{-1} cell protein, 0.32 ± 0.04 nmol mg^{-1} cell protein, 0.88 ± 0.04 nmol mg^{-1} cell protein, 0.42 ± 0.09 nmol mg^{-1} cell protein and 0.39 ± 0.02 nmol mg^{-1} cell protein, respectively.

4 h incubation, such that, after 24 h, levels of cicaprost-induced cAMP generation were reduced by 67.9±6.7, 76.9±11.8 and 62±3.7% at 24 h in HEK.hIP, HEK.mIP and HEL cells, respectively, relative to the levels generated in vehicle-treated cells (data not shown).

To investigate if the observed reductions in IP-mediated signalling may be due to overall reductions in the level of IP expression rather than due to its functional impairment to mediate G protein/effector coupling *per se*, the effects of R115777 on the radioligand-binding properties of IP were investigated. Pre-incubation of cells with R115777 even at concentrations as high as 1 μM for 16 h did not significantly affect [^{3}H]iloprost binding by IP receptors expressed in HEK.hIP, HEK.mIP or in HEL cells (Table 1). Consistent with the above data, [^{3}H]CGP-12177 binding by the β_2AR overexpressed in HEK.293 cells was also unaffected by R115777 (Table 1).

Effect of R115777 on IP-mediated [Ca^{2+}]$_i$ mobilization

The effect of R115777 on IP coupling to Gα_q/phospholipase C (PLC)β activation was also examined by analysing cicaprost-induced [Ca^{2+}]$_i$ mobilization by the hIP and mIP expressed in HEK.hIP and HEK.mIP cells, respectively, and in HEL cells. HEK 293 cells overexpressing the α isoform of the human thromboxane A_2 (TXA_2) receptor (TPα) served as control for a nonisoprenylated G_q/PLC-coupled receptor. Stimulation of HEK.hIP (Figure 2a; $\Delta[Ca^{2+}]_i = 105 \pm 12$ nM) and HEK.mIP (Figure 2c; 204±25 nM) cells with cicaprost (1 μM) each induced significant transient rises in [Ca^{2+}]$_i$ levels. In contrast, pretreatment of cells with R115777 (1 μM) for 16 h significantly reduced [Ca^{2+}]$_i$ mobilization both in HEK.hIP cells (Figure 2a; $\Delta[Ca^{2+}]_i = 41.4 \pm 4.9$ nM, $P = 0.0014$) and in HEK.mIP cells (Figure 2c; $\Delta[Ca^{2+}]_i = 86.7 \pm 15$ nM, $P = 0.0096$). From concentration–response studies, IC_{50} values were 40 nM R115777 in HEK.hIP cells (Figure 2b) and 65 nM R115777 in HEK.mIP cells (Figure 2d), respectively, while the extents of maximal inhibition of cicaprost-induced [Ca^{2+}]$_i$ mobilization using 1 μM R115777 were 60.3±4.4 and 50.9±8.2% for the hIP and mIP, respectively, relative to that mobilized in the absence of R115777 (Figure 2a and c). Stimulation of hIPs expressed in HEL cells also mediated significant transient rises in [Ca^{2+}]$_i$ mobilization in response to cicaprost (Figure 2e; $\Delta[Ca^{2+}]_i = 74.1 \pm 11.4$ nM). Moreover, pre-incubation of HEL cells with 1 μM R115777 for 16 h significantly reduced cicaprost-mediated [Ca^{2+}]$_i$ mobilization (Figure 2e; $\Delta[Ca^{2+}]_i = 28.3 \pm 8.2$ nM, $P = 0.047$), being maximally reduced by 58.3±11.4% of that observed in the absence of R115777 (Figure 2e and f). An IC_{50} value for inhibition of cicaprost signalling through the hIP expressed in HEL cells was 37.7 nM R115777, which was not significantly different from that observed for the hIP expressed in HEK.hIP cells. In contrast, while stimulation of HEK.TPα cells with the TXA_2 mimetic U46619 (1 μM) mediated efficient rises in [Ca^{2+}]$_i$ mobilization (Figure 2g; $\Delta[Ca^{2+}]_i = 28.3 \pm 8.2$ nM), pre-incubation of cells with R115777 had no effect whatsoever on either the ability of TPα to bind its radioligand [^{3}H]SQ29,548 (Table 1) or to mobilize [Ca^{2+}]$_i$ even at concentrations as high as 1 μM R115777 (Figure 2g and h; compare $\Delta[Ca^{2+}]_i = 196 \pm 16.0$ nM *versus* $\Delta[Ca^{2+}]_i = 192 \pm 24.5$ nM, $P = 0.92$).

Table 1 Radioligand-binding assays

Cell type	*R115777 (1 μM)*	*[^{3}H]Iloprost bound*[a] *(fmol mg^{-1} protein)*
HEK.hIP	−	1790±199
HEK.hIP	+	1934±310
HEK.mIP	−	1703±189
HEK.mIP	+	1808±125
HEL	−	77.7±7.75
HEL	+	74.5±4.88
HEK 293	−	16.0±0.52
HEK 293	+	12.3±0.23
Cell type	*R115777 (1 μM)*	*[^{3}H]CGP-12177 bound*[b] *(fmol mg^{-1} protein)*
HEK.β_2AR	−	334±35.3
HEK.β_2AR	+	325±47.7
HEK 293	−	10.0±0.78
HEK 293	+	9.20±0.03
Cell type	*R115777 (1 μM)*	*[^{3}H]SQ29,548 Bound*[c] (fmol mg^{-1} protein)
HEK.TPα	−	2420±266
HEK.TPα	+	2404±80.8
HEK.TPβ	−	1602±140
HEK.TPβ	+	1716±93.9
HEK 293	−	12.8±0.30
HEK 293	+	14.5±3.57

[a]Radioligand-binding assays were performed on crude cell membrane fractions ($P100$; 50–100 μg/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 μg per assay) in the presence of 25 nM [^{3}H]CGP-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 mean±s.e.m ($n = 3$).

Effect of R115777 on IP receptor-mediated desensitization of TP signalling

Previously, it has been established that signalling by the TPα, but not by the TPβ, isoform of the human TXA_2 receptor is subject to IP-mediated counter-regulation or heterologous desensitization through a mechanism involving direct protein kinase (PK) A phosphorylation of TPα (Walsh *et al*., 2000). Thus, we next investigated the effect of R115777 on IP-mediated desensitization of TP receptor responses. Both TPα and TPβ receptors stably expressed in HEK 293 cells (HEK.TPα and HEK.TPβ cells, respectively) exhibited efficient mobilization of [Ca^{2+}]$_i$ in response to stimulation with the TXA_2 mimetic U46619 (Figure 3a and b; $\Delta[Ca^{2+}]_i = 172 \pm 21.8$ nM for TPα; $\Delta[Ca^{2+}]_i = 139 \pm 8.99$ nM for TPβ, respectively). While cicaprost (1 μM) did not induce a measurable increase in [Ca^{2+}]$_i$ mobilization in HEK.TPα cells *per se*, it significantly reduced [Ca^{2+}]$_i$ mobilization in response to subsequent stimulation of cells with U46619 (Figure 3a; $\Delta[Ca^{2+}]_i = 36.0 \pm 3.05$ nM, $P \leqslant 0.0035$). In contrast, U46619-mediated signalling by HEK.TPβ cells was unaffected by cicaprost (Figure 3b; $\Delta[Ca^{2+}]_i = 121 \pm 6.10$ nM; $P = 0.117$). While pre-incubation of HEK.TPα cells with R115777 (1 μM) had no significant effect on U46619-induced [Ca^{2+}]$_i$ mobilization (Figure 3c), R115777 significantly impaired the level of cicaprost-induced desensitization of TPα signalling relative to vehicle-treated cells (Figure 3c; $\Delta[Ca^{2+}]_i = 119 \pm 4.77$ nM, $P \leqslant 0.0001$), restoring U46619-mediated [Ca^{2+}]$_i$ mobilization to 71.7±10.9% of that originally observed in the absence of

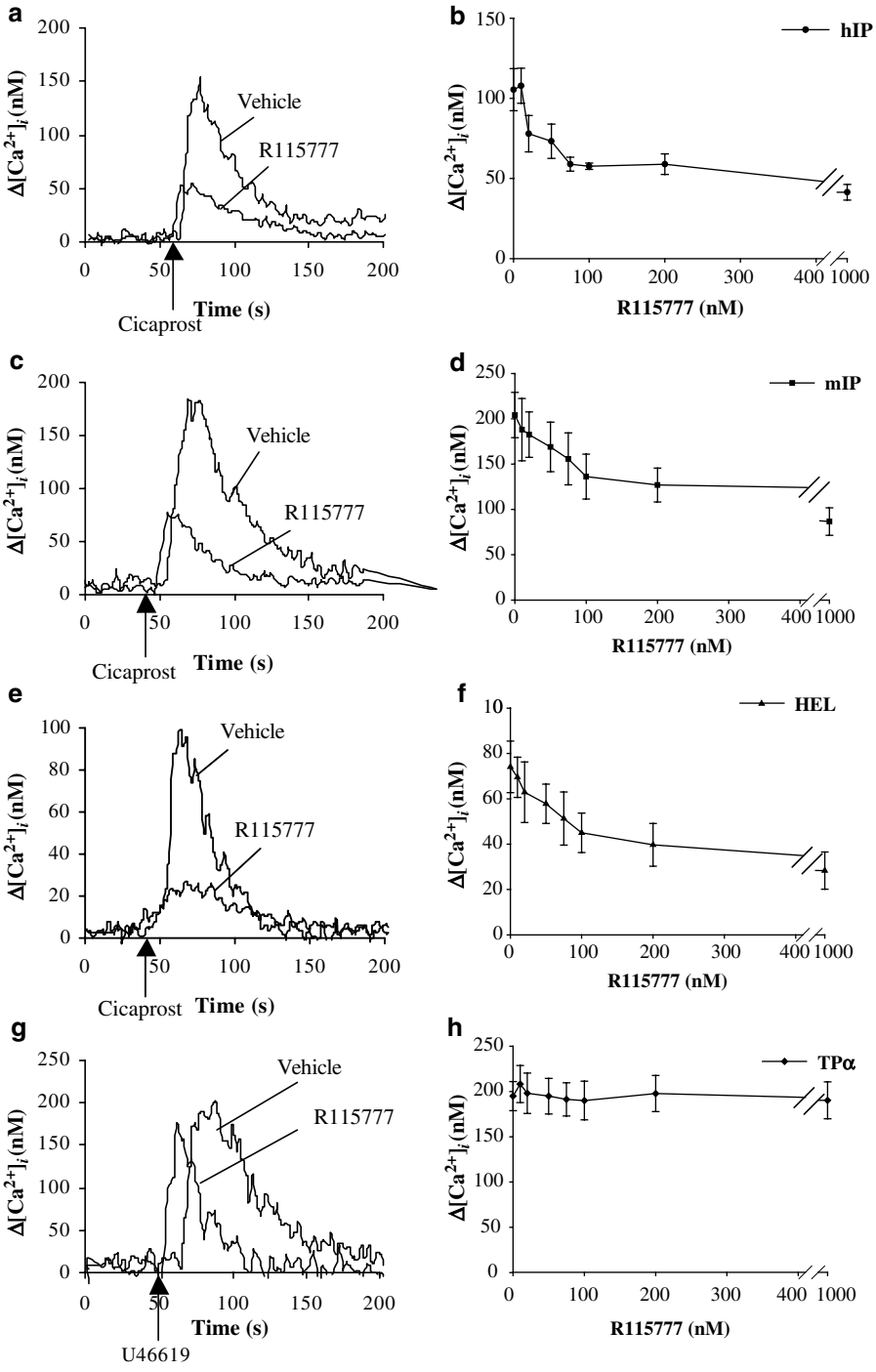

Figure 2 Effect of R115777 on IP-mediated $[Ca^{2+}]_i$ mobilization. HEK.hIP cells (panels a and b), HEK.mIP cells (panels c and d), HEL cells (panels e and f) and, as controls, HEK.TPα cells (panels g and h) were pre-incubated with either 1000 nM R115777 (R115777) or with 0.0001% DMSO (Vehicle) (panels a, c, e and g) or, alternatively, with 0–1000 nM R115777 (panels b, d, f and h) for 16 h prior to harvesting. Cells, preloaded with Fura2/AM, were stimulated with 1 μM cicaprost (panels a, c and e) or with 1 μM U46619 (panel g) at times indicated by the arrows. Data presented in panels a, c, e and g are representative of at least four independent experiments and are plotted as changes in $[Ca^{2+}]_i$ mobilization ($\Delta[Ca^{2+}]_i \pm$ s.e.m. nM, $n=4$) as a function of time (s). In panels b, d, f and h, mean changes in $[Ca^{2+}]_i$ mobilization ($\Delta[Ca^{2+}]_i \pm$ s.e.m. nM, $n=4$) are plotted as a function of R115777 concentration (nM).

cicaprost (Figure 3c and e). From concentration–response studies, an IC_{50} value for inhibition of cicaprost desensitization of TPα signalling was 131 nM R115777 (data not shown). Consistent with previous reports (Walsh *et al*., 2000; Lawler *et al*., 2001), U46619-mediated signalling by HEK.TPβ cells was unaffected by cicaprost irrespective of pre-incubation of cells with or without R115777 or its concentration (Figure 3b and d; data not shown; $P>0.5$).

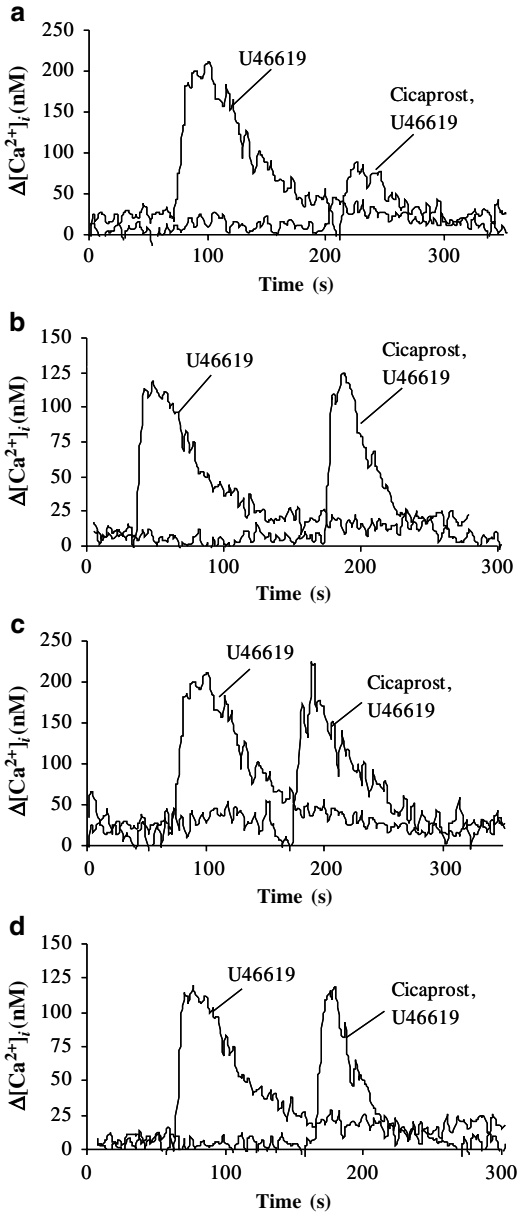

Figure 3 Effect of R115777 on IP-mediated desensitization of TP-mediated signalling. HEK.TPα cells (panels a and c) and HEK.TPβ cells (panels b and d), transiently transfected with pCMV:$G\alpha_q$, were pre-incubated with either the vehicle 0.0001% DMSO (panels a and b) or with 1000 nM R115777 (panels c and d) for 16 h prior to harvesting. Cells were preloaded with Fura2/AM and were stimulated with 1 μM U46619 at 50 s (U46619) or with 1 μM cicaprost at 50 s, followed by 1 μM U46619 at 150 s–200 s, approximately (Cicaprost, U46619). Data presented in panels a–d are each representative of four independent experiments and are plotted as changes in $[Ca^{2+}]_i$ mobilization ($\Delta[Ca^{2+}]_i \pm$ s.e.m. nM, $n=4$) as a function of time (s).

Effect of R115777 on HDJ-2 isoprenylation

HDJ-2 (DNA J homologue), a molecular co-chaperone protein, is farnesylated at Cys^{394} within its carboxyl-terminal CAAX motif (Kanazawa *et al*., 1997). To compare the relative inhibitory effects of R115777 on the isoprenylation of the IP to that of the HDJ-2, we examined the effect of R115777 on the farnesylation-dependent proteolytic processing of HDJ-2

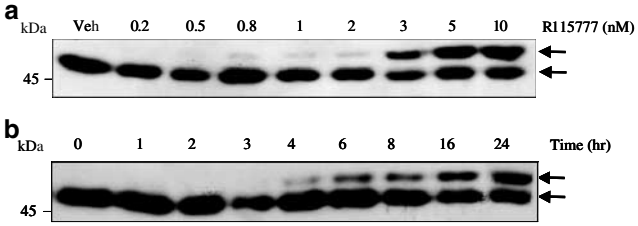

Figure 4 Effect of R115777 on isoprenylation and processing of HDJ-2. Panel a: HEK 293 cells were pre-incubated for 24 h with vehicle 0.0001% DMSO (Veh) or with 0–10 nM R115777. Panel b: HEK 293 cells were pre-incubated for 0–24 h with vehicle 0.0001% DMSO (Veh) or with 5 nM R115777. Thereafter, cells were harvested and aliquots (25 μg) of whole-cell protein (25 μg) were resolved by SDS–PAGE/immunoblotting and screened with the *anti*-HDJ-2 antibody as described in '*Methods*'. The arrows to the right of panels a and b indicate the slower migrating nonfarnesylated species (~49 kDa) and the faster migrating, farnesylated species of HDJ-2 (~45–46 kDa), while the position of the 45 kDa molecular weight marker is indicated to the left.

expressed in HEK 293. Pre-incubation of HEK 293 cells with R115777 (0–10 nM) for 24 h significantly impaired HDJ-2 farnesylation and processing, as evidenced by the accumulation of the nonfarnesylated 49 kDa form of HDJ-2 in whole-cell lysates (Figure 4a). The IC_{50} was determined to be 4.5 nM R115777, while the minimum inhibitory concentration (mIC) for the appearance of the slower migrating nonfarnesylated form was detected at 0.5 nM (Figure 4a; data not shown).

In time-course assays, similar to that of the IP signalling data, R115777 (5 nM) significantly affected the mobility of HDJ-2 after ~3–4 h and continued to 24 h, as evidenced by the accumulation of the nonfarnesylated 49 kDa form of HDJ-2 (Figure 4b; data not shown).

Effect of R115777 on protein isoprenylation in vitro *and* in vivo

To directly establish whether farnesylation of the CAAX motif of the IP, with the sequence -CSLC, is efficiently inhibited by R115777 and to establish whether, like that of N-Ras and Ki-Ras (Whyte *et al*., 1997), it may undergo alternative geranylgeranylation such as in conditions of FTase inhibition, we developed an *in vitro* assay approach to directly compare isoprenylation of the CAAX (-CSLC) motif of IP to those of -CVLS ($Ha\text{-}Ras^{WT}$), -CVIM (Ki-Ras) and -CVLL (Rac 1, a known substrate for GGTase 1). To this end, four variants of Ha-Ras were created by directly replacing its -CAAX motif sequence (-CVLS, corresponding to residues 186–189) with those of the IP ($Ha\text{-}Ras^{CSLC}$), the nonisoprenylated IP control sequence ($Ha\text{-}Ras^{SSLC}$), the geranylgeranylated Rac 1 sequence ($Ha\text{-}Ras^{CVLL}$) and the alternatively isoprenylated Ki-Ras sequence ($Ha\text{-}Ras^{CVIM}$). Thereafter, the respective *in vitro* transcripts were translated *in vitro* in a rabbit reticulocyte lysate system either in the presence of [^{35}S]methionine or, to examine the effect of R115777 on the isoprenylation, in the presence of the isoprene precursor [^{3}H]MVA) that can be readily converted into the isoprene donors FPP and GGPP by the reticulocyte lysate system (Kinsella *et al*., 1991a, b), or alternatively in the presence of [^{3}H] FPP or [^{3}H] GGPP.

Based on the incorporation of [^{35}S]methionine, all Ha-Ras variants were synthesized *in vitro* with comparable efficiencies, while the level of endogenous protein synthesis observed in the absence of exogenous RNA was negligible (Figure 5a; compare lanes 1–5 *versus* lane 6). Moreover, in the presence of [^{3}H]MVA, single protein bands of approximately 21 kDa, corresponding to isoprenylated forms Ha-Ras^{WT}, Ha-Ras^{CSLC}, Ha-Ras^{CVLL} and Ha-Ras^{CVIM}, were readily detected, albeit at different efficiencies of labelling (Figure 5b; lanes 1, 2, 4 and 5), while no [^{3}H]MVA was found associated with Ha-Ras^{SSLC}, confirming that mutation of the critical Cys^{186} to Ser^{186} within the 'C^{186}SLC' motif of Ha-Ras^{CSLC} obliterates isoprenylation (Figure 5b; lane 3). Similar to that observed with the [^{35}S]methionine-labelled reactions, the level of [^{3}H]MVA incorporated into endogenous reticulocyte proteins was negligible in the absence of exogenous RNA (Figure 5b; lane 6). Inclusion of R115777 (25 nM; or 1 μM, data not shown) during the *in vitro* translation completely inhibited [^{3}H]MVA incorporation into both Ha-Ras^{WT} and Ha-Ras^{CSLC} (Figure 5c; lanes 1 and 2), but had no effect on the isoprenylation of GGTase 1 control substrate Ha-Ras^{CVLL} (Figure 5c; lane 4). Moreover, R115777 had no significant effect on the level of [^{3}H]MVA incorporation into Ha-Ras^{CVIM} (Figure 5c; lane 5), consistent with previous reports that Ki-Ras can be alternatively isoprenylated by GGTase 1 in the presence of FTase inhibitors (James *et al*., 1995; Whyte *et al*., 1997; Zhang *et al*., 1997). Indeed, incorporation of the [^{3}H]MVA label into Ha-Ras^{CVIM} was not substantially inhibited by even increasing the concentration of R115777 to 1 μM (data not shown).

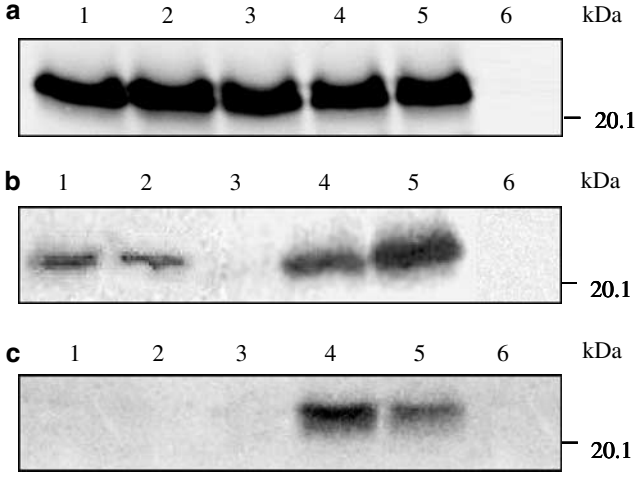

Figure 5 Effect of R115777 on isoprenylation of the -CSLC motif *in vitro*. RNA transcripts coding for Ha-Ras (lane 1), Ha-Ras^{CSLC} (lane 2), Ha-Ras^{SSLC} (lane 3), Ha-Ras^{CVLL} (lane 4) and Ha-Ras^{CVIM} (lane 5) were translated *in vitro* in the presence of [^{35}S]methionine (panel a), [^{3}H]MVA (panel b) or [^{3}H]MVA plus 25 nM R115777 (panel c) as described under 'Methods'. Parallel *in vitro* translations carried out in the absence of exogenous added RNA served as controls for [^{35}S]methionine and [^{3}H]MVA reactions, respectively (panels a–c, lane 6). Aliquots from each translation reaction (8 μl from [^{35}S]methionine reactions and 25 μl from [^{3}H]MVA reactions) were resolved by SDS–PAGE and electroblotted onto PVDF membranes. Thereafter, blots were subject to fluorography to visualize the radiolabeled proteins. Blots containing ^{35}S-labelled proteins (panel a) were exposed for 16 h, while those containing ^{3}H-labelled proteins (panels b and c) were exposed for 21 days. The position of 20.1 kDa molecular weight marker (kDa) is indicated to the right of panels a–c.

When *in vitro* translations were carried out in the presence of [^{3}H]FPP, Ha-Ras^{WT}, Ha-Ras^{CSLC} and Ha-Ras^{CVIM} (Figure 6b; lanes 1, 2 and 4) were efficiently labelled, while Ha-Ras^{CVLL} was not farnesylated (Figure 6b; lane 3) and no label was incorporated into endogenous proteins in the absence of added RNA (Figure 6b; lane 5). Moreover, R115777 (25 nM) completely inhibited farnesylation of Ras^{WT}, Ha-Ras^{CSLC} and Ha-Ras^{CVIM} (Figure 6d; lanes 1, 2 and 4). Ha-Ras^{CVIM} was also efficiently labelled with [^{3}H]GGPP, as was the GGTase 1 control substrate Ha-Ras^{CVLL} (Figure 6c; lanes 4 and 3, respectively) and R115777 did not substantially affect geranylgeranylation of either Ras^{CVLL} or Ras^{CVIM} (Figure 6e; lanes 3 and 4, respectively). On the other hand, neither Ha-Ras^{WT} or Ha-Ras^{CSLC} was labelled with [^{3}H]GGPP (Figure 6c; lanes 1 and 2) and R115777 had no effect on labelling in the presence of [^{3}H]GGPP (Figure 6e).

Thereafter, to investigate whether R115777 inhibits isoprenylation of the -CSLC motif of the IP *in vivo*, whole-cell metabolic labelling studies were carried out in the presence of the [^{3}H]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 pHM6 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 (Figure 7). In the absence of R115777, there was efficient protein isoprenylation, as evidenced by the incorporation of [^{3}H]MVA-derived isoprene units into a host of cell proteins (Figure 7a). Moreover, following immunoprecipitation with *anti*-HA antibody, it was evident that Ha-Ras^{WT}, Ha-Ras^{CSLC}, Ha-Ras^{CVLL} and Ha-Ras^{CVIM} were efficiently isoprenylated *in vivo*, albeit at different efficiencies of labelling (Figure 7c; lanes 1, 2, 4 and 5), while no [^{3}H]MVA was found associated with Ha-Ras^{SSLC} immunoprecipitates (Figure 7c; lane 3) or with immunopreci-

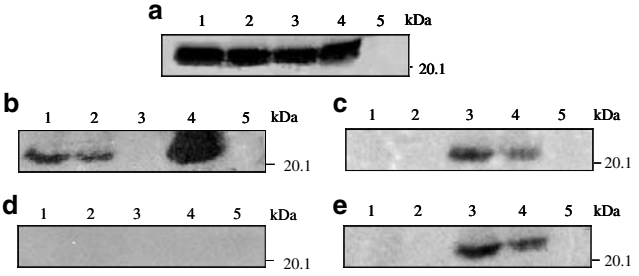

Figure 6 Effect of R115777 on incorporation of [^{3}H]FPP and [^{3}H]GGPP into Ras translation products. RNA transcripts coding for Ha-Ras (lane 1), Ha-Ras^{CSLC} (lane 2), Ha-Ras^{CVLL} (lane 3) and Ha-Ras^{CVIM} (lane 4) were translated *in vitro* in the presence of [^{35}S]methionine (panel a), [^{3}H]FPP (panel b), [^{3}H]GGPP (panel c), [^{3}H]FPP plus 25 nM R115777 (panel d), or [^{3}H]GGPP plus 25 nM R115777 (panel e) as described under 'Methods'. Parallel *in vitro* translations carried out in the absence of exogenous added RNA served as controls for [^{35}S]methionine, [^{3}H]FPP and [^{3}H]GGPP reactions, respectively (panels a–e, lane 6). Aliquots from each translation reaction (8 μl from [^{35}S]methionine reactions and 25 μl from [^{3}H]FPP and [^{3}H]GGPP reactions) were resolved by SDS–PAGE and electroblotted onto PVDF membranes. Thereafter, blots were subject to fluorography to visualize the radiolabelled proteins. Blots containing ^{35}S-labelled proteins were exposed for 16 h (panel a), while those containing ^{3}H-labelled proteins were exposed for 21 days (panels b–e). The position of 20.1 kDa molecular weight marker (kDa) is indicated to the right of panels a–e.

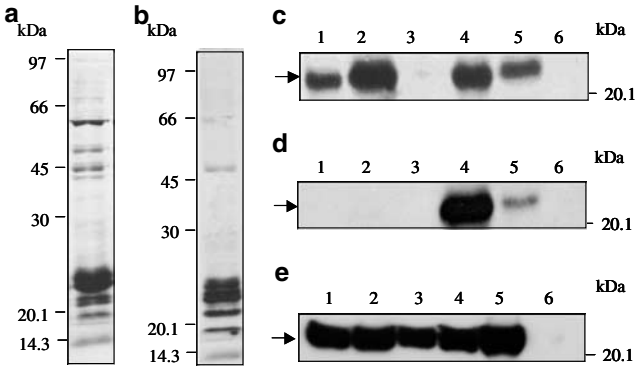

Figure 7 Effect of R115777 on the isoprenylation of the -CSLC motif in whole cells. HEK 293 cells were transiently co-transfected with pMEV along with either pHM:Ras^{WT}, pHM:Ras^{CSLC}, pHM:Ras^{SSLC}, pHM:Ras^{CVLL}, pHM:Ras^{CVIM} or, as a negative control, with the empty vector pHM6 prior to metabolic labelling with [^{3}H]MVA either in the absence (panels a and c) or presence (panels b and d) of R115777, respectively. Panels a and b: to analyse the effect of R115777 on total metabolic labelling, aliquots (60 μg) of whole-cell proteins isolated from cells labelled in the absence (panel a) or presence of 1 μM R115777 (panel b) were resolved directly by SDS–PAGE, followed by electroblotting onto PVDF, where proteins isolated from the mock (pHM6) transfected HEK 293 cells are shown as representatives in panels a and b. Panels c and d: Alternatively, cell lysates from the respective transfected cells (containing approximately 500 μg protein/lysate) were subject 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), 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 onto PVDF membrane. Blots in panels a–d were then soaked in Amplify for 30 min and exposed to X-Omat AR 5 film for 21 days at -70°C. Panel e: In parallel experiments, HA-tagged proteins immunoprecipitated from nonmetabolically 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, with pHM6 (lane 6) were resolved by SDS–PAGE, followed by electroblotting onto PVDF membrane; thereafter, membranes were screened using the peroxidase-conjugated *anti*-(HA 3F10) serum, followed by chemiluminescent detection. The positions of the molecular weight markers (kDa) are indicated either to the left or right of panels a–e, and the positions of the isoprenylated 21.4 kDa protein corresponding to Ha-Ras, Ha-Ras^{CSLC}, Ha-Ras^{SSLC}, Ha-Ras^{CVLL} and Ha-Ras^{CVIM} are indicated by *arrows* (panels c–e).

pitates from mock-transfected control cells (Figure 7c; lane 6). Inclusion of R115777 (1 μM) during the metabolic labelling reduced the level of [^{3}H]MVA incorporation into a range of cell proteins (Figure 7b), particularly into several larger isoprenylated proteins greater than 45 kDa that were previously established to be farnesylated proteins (Reese & Maltese, 1991). While R115777 completely inhibited isoprenylation of Ha-Ras^{WT} and Ha-Ras^{CSLC} (Figure 7d; lanes 1 and 2), it did not affect isoprenylation of the GGTase 1 substrate Ha-Ras^{CVLL} (Figure 7d; lane 3) and reduced, but did not abolish, isoprenylation of Ha-Ras^{CVIM} (Figure 7d; lane 4). In parallel experiments, the identities of the precipitated/isoprenylated proteins to be those of the HA-tagged Ha-Ras^{WT}, Ha-Ras^{CSLC}, Ha-Ras^{SSLC}, Ha-Ras^{CVLL} and Ha-Ras^{CVIM} were each confirmed by immunoprecipitation with the *anti*-HA 101R antibody, followed by direct screening of resultant Western blots with peroxidase-conjugated *anti*-HA 3F10 antibody (Figure 7e). Hence, taken together, these data confirm that the FTase inhibitor R115777 fully impairs isoprenylation of both Ha-Ras^{WT} and Ha-Ras^{CSLC} *in vitro* and in whole cells, and like that of the -CVLS motif associated with Ha-Ras^{WT}, the -CSLC motif associated with the IP does not undergo alternative geranylgeranylation in the presence of R115777 and does not act a substrate for GGtase 1 *in vitro* or *in vivo*. On the other hand, while R115777 inhibited farnesylation of the variant Ha-Ras^{CVIM}, its -CVIM motif supported alternative geranylgeranylation in the presence of strong FTase inhibition, serving as a substrate for GGtase 1 both *in vitro* and *in vivo*, consistent with previous reports (James *et al*., 1995; Whyte *et al*., 1997; Zhang *et al*., 1997).

Discussion

Prostacyclin plays a key role in vascular haemostasis (Vane & Botting, 1995), but is also a potent pain and pro-inflammatory mediator (Murata *et al*., 1997); it confers a cytoprotective effect against tissue injury during acute myocardial ischaemia (Sakai *et al*., 1990; Xiao *et al*., 2001) and enhances endothelial cell survival and/or endothelial cell proliferation supporting neovascularization and angiogenesis (Zachary, 2001). We have recently established that both the mouse (m) and human (h) IP are somewhat unique among GPCRs in that they are isoprenylated through farnesylation (Hayes *et al*., 1999; Miggin *et al*., 2002). While disruption of isoprenylation had no affect on ligand binding, it was found to be critical for IP signalling and function (Hayes *et al*., 1999; Lawler *et al*., 2001; Miggin *et al*., 2002). Herein, we present evidence that R115777, a FTase inhibitor, is a potent and selective inhibitor of IP farnesylation *in vitro* and *in vivo*. R115777 inhibited IP farnesylation at sub-nanomolar concentrations, concentrations that are likely to be found in the circulation, as documented by early clinical trials (End *et al*., 2001; Crul *et al*., 2002).

Owing to the high incidence of human tumours harbouring oncogenic Ras mutants, interrupting the Ras-signalling pathway represents a major strategy in the development of novel chemotherapeutics (Adjei, 2001). Recently, much attention has focused on the prevention of membrane localization of Ras through inhibition of its farnesylation by FTase (Rowinsky *et al*., 1999). The specificity of such inhibitors relies on the fact that most isoprenylated proteins are modified by C-20 geranylgeranyl groups rather than C-15 farnesyl groups and, thus, FTase inhibitors exploit the necessary farnesylation of Ras proteins without targeting GGTase I or II substrates (Adjei, 2001). R115777 (Zarnestra) is an orally active imidazole-containing methylquinone that acts as a potent and selective competitive inhibitor of the CaaX peptide-binding site of FTase (End, 1999). Although originally developed as Ras inhibitors, numerous studies have demonstrated that FTase inhibitors may target a variety of other proteins, particularly farnesylated protein substrates (Cox & Der, 1997; Gibbs & Oliff, 1997; Oliff, 1999; Karp *et al*., 2001a). Thus, in view of the fact that the human (h) and mouse (m) IPs are farnesylated and therefore are potential '*nonintended*' targets for FTase inhibitors, in the current study we investigated the effect of R115777 on isoprenylation and intracellular signalling by both the mIP and hIP and sought to

establish whether the IPs are true targets for R115777 both *in vitro* and *in vivo* (whole cells).

Pre-incubation of HEK.hIP, HEK.mIP and HEL cells with R115777 yielded time- and concentration-dependent reductions in cAMP generation ($IC_{50} = 0.41$, 0.37 and 0.60 nM R115777, respectively) and $[Ca^{2+}]_i$ mobilization ($IC_{50} = 40$ nM, , 65 nM and 38 nM R115777, respectively), but had no effect on ligand binding or on signalling by the nonisoprenylated β_2AR or TPα, confirming that the observed effects of R115777 are targeted to the IP itself and not to another nonspecific component of the signalling system. It is noteworthy that the IC_{50} for inhibition of IP-mediated cAMP generation was substantially lower compared to those previously reported for the purified FTase in *in vitro* enzyme inhibition assays (End *et al*., 2001; Karp *et al*., 2001b; Kelland *et al*., 2001; Norman, 2002), suggesting that the IP is particularly sensitive to FTase inhibition by R115777. It was also noteworthy that the IC_{50} values for inhibition of cicaprost-induced cAMP generation were significantly less than those for inhibition of $[Ca^{2+}]_i$ mobilization. These data are entirely consistent with previous data whereby IP coupling to the Gs/adenylyl cyclase effector system was found to be much more functionally dependent on isoprenylation than was IP coupling to the Gq/PLCβ effector system (Hayes *et al*., 1999; Lawler *et al*., 2001; Miggin *et al*., 2002).

Intermolecular crosstalk has been widely documented to occur between the antiaggregatory adenylyl cyclase/PKA system modulated by prostacyclin and the proaggregatory PLCβ/PKC system modulated by TXA_2 in platelets and vascular smooth muscle (Coleman *et al*., 1994; Cheng *et al*., 2002). More specifically, it was recently established that the TPα, but not TPβ, isoform of the human TXA_2 receptor undergoes IP-induced cross-desensitization through a mechanism involving direct PKA phosphorylation of TPα (Walsh *et al*., 2000). Given the central role of the latter counter-regulation between IP : TP signalling within the vasculature, coupled to the finding that R115777 is a potent inhibitor of IP function, herein we also examined the effect of R115777 on IP-mediated desensitization of TP responses. Both TPα and TPβ exhibited efficient U46619-induced $[Ca^{2+}]_i$ mobilization and, consistent with previous reports (Walsh *et al*., 2000), cicaprost reduced $[Ca^{2+}]_i$ mobilization by Pα but not by TPβ. Pre-incubation with R115777 significantly reduced the level of cicaprost-induced cross-desensitization of TPα but had no effect on signalling by or cross-desensitization of TPβ. Hence, these data further confirm the functional requirement for isoprenylation by the IP and indicate that impairment of isoprenylation by FTase inhibition is also likely to have downstream effects on other signalling systems that are either directly or indirectly linked to IP signalling.

Given that FTase inhibitors are reversible, direct measurement of FTase activity as a marker of drug effect has proved difficult and studies have therefore focused on the detection of altered farnesylation of surrogate FTase substrates (Adjei *et al*., 2000). Much attention has focused on prelamin A and on HDJ-2, both of which are farnesylated (Adjei *et al*., 2000; End *et al*., 2001). The ubiquitously expressed molecular chaperone HDJ-2 undergoes a readily detectable mobility shift upon treatment with FTase inhibitors (Kanazawa *et al*., 1997; Adjei *et al*., 2000) and therefore serves as a convenient assay to assess the relative sensitivities of various farnesylated substrates, such as the IP herein, to FTase inhibitors. R115777 significantly impaired the isoprenylation and proteolytic processing of HDJ-2 in HEK 293 cells in a time- and concentration-dependent manner (mIC = 0.5 nM R115777), consistent with previous reports in other cell types (Adjei *et al*., 2000; Karp *et al*., 2001a, b). Moreover, given that the IC_{50} for inhibition of cicaprost-induced cAMP generation reported herein ranged from 0.41 to 0.60 nM R115777 and that the IC_{50} for inhibition of HDJ-2 isoprenylation was 4.5 nM, it appears that IP isoprenylation and agonist-induced cAMP generation is 10-fold more sensitive to R115777 inhibition than is HDJ-2.

Major regressions have been observed in the treatment of patients with leukaemia and a variety of solid neoplasms, many of which are associated with a high incidence of Ki-ras mutations. However, there is substantial evidence suggesting that, in the presence of FTase inhibitors, both Ki-Ras and N-Ras may become alternatively geranylgeranylated (James *et al*., 1995; Whyte *et al*., 1997; Zhang *et al*., 1997), thereby compensating for any potential benefits gained through inhibition of Ki- or N-Ras farnesylation and therefore possibly accounting for such regressions. In view of the latter, we next sought to establish whether R115777 could inhibit IP isoprenylation *in vitro* and in whole cells (*in vivo*) and to determine whether, in the presence of strong FTase inhibition, the IP undergoes alternative geranylgeranylation similar to that of Ki-Ras and N-Ras. Initially, we generated a series of chimeric Ha-Ras mutants in which we directly replaced its -CaaX motif (-CVLS) with that of IP (Ha-Ras^{CSLC}), the nonisoprenylated IP control sequence (Ha-Ras^{SSLC}), the geranylgeranyled Rac 1 sequence (Ha-Ras^{CVLL}) or the alternatively isoprenylated Ki-Ras sequence (Ha-Ras^{CVIM}). Based on the incorporation of [^{35}S]methionine, all proteins were synthesized *in vitro* with comparable efficiencies, while Ha-Ras^{WT}, Ha-Ras^{CSLC}, Ha-Ras^{CVLL} and Ha-Ras^{CVIM}, but not Ha-Ras^{SSLC}, proteins were also efficiently isoprenylated both *in vitro* and in whole cells (*in vivo*). In the presence of R115777, Ha-Ras^{WT} and Ha-Ras^{CSLC} isoprenylation was completely inhibited, suggesting that, unlike Ki-Ras and N-Ras, the C-terminal CaaX motifs of both Ha-Ras (-CVLS) and IP (-CSLC) do not undergo alternative isoprenylation in the presence of R115777 *in vitro* or *in vivo*. On the other hand, R115777 did not inhibit isoprenylation of the GGTase 1 substrate Ha-Ras^{CVLL} (Rac 1 sequence) or the Ha-Ras^{CVIM} (Ki-Ras sequence), confirming that Ha-Ras^{CVIM} can become alternatively geranylgeranylated, serving as a GGTase 1 substrate in the presence of the FTase inhibitor R115777. In the presence of [^{3}H]FPP, in agreement with previous data, Ha-Ras^{WT}, Ha-Ras^{CSLC}, Ha-Ras^{CVIM}, but not Ha-Ras^{CVLL}, were each efficiently farnesylated *in vitro* and inclusion of R115777 completely inhibited farnesylation of Ha-Ras^{WT}, Ha-Ras^{CSLC} and Ha-Ras^{CVIM}. In the presence of [^{3}H]GGPP, Ha-Ras^{CVLL} (Rac 1 sequence) and Ha-Ras^{CVIM} (Ki-Ras sequence) were geranylgeranylated, while R115777 had no effect on labelling of either Ha-Ras^{CVLL} or indeed of Ha-Ras^{CVIM}, further confirming that, while R115777 inhibits farnesylation of the variant Ha-Ras^{CVIM}, in the presence of strong FTase inhibition it can become alternatively geranylgeranylated and, thus, can serve as a GGtase 1 substrate.

The recent reevaluation of the importance of the IP *in vivo*, such as through IP knockout studies in mice, have confirmed the critical role of prostacyclin as an endogenous *anti*-platelet, *anti*-thrombotic and pro-inflammatory mediator in the setting of vascular injury (Murata *et al*., 1997; Cheng *et al*., 2002). In

view of the essential nature of isoprenylation on IP signalling, data presented herein strongly suggest that the use of R115777 may confer important modulatory effects on IP function and, in turn, on the physiologic processes in which prostacyclin and its receptor are implicated. Moreover, *in vitro* and whole-cell data presented herein indicate that the IP is particularly sensitive to inhibition by the FTase inhibitor R115777 and that it has a strict requirement for farnesylation, failing to be alternatively geranylgeranylated. Bearing this in mind, it is indeed tempting to caution that further clinical trials involving FTase inhibitors, such as R115777, should be so designed to investigate their effects on other farnesylated proteins, such as the IP, not directly implicated as direct anti-Ras/anti-tumour targets.

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