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Original Paper

XR 3054, Structurally Related to Limonene, is a Novel Inhibitor of Farnesyl Protein Transferase

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In this report, a novel inhibitor of farnesyl protein transferase (FPTase) is described. The compound, XR 3054, is structurally similar to farnesol, a component of the reaction in which FPTase catalyses transfer of farnesol pyrophosphate to the CAAX recognition motif on proteins. The compound was selected initially because of its ability to inhibit *in vitro* farnesylation of CAAX recognition peptides with an IC_{50} of 50 μM. The farnesylation of p21 ras was reduced in a dose-dependent manner in the presence of XR 3054. Similarly XR 3054 was able to reduce the anchorage-independent growth of V12 H-*ras* transformed NIH 3T3 cells in a focus formation assay in soft agar, with an IC_{50} value of 30 μM, whilst not affecting the anchorage-independent growth of v-*raf* transformed cells. XR 3054 reduced the phosphorylation of p42 mitogen activated protein (MAP) kinase in parental NIH 3T3 cells and V12 H-*ras* transformed NIH 3T3 cells, but constitutively active v-*raf* transformed cells showed no reduction in phosphorylation of ERK2 in the presence of XR 3054. XR 3054 inhibited the proliferation of the prostatic cancer cell lines LnCAP and PC3 and the colon carcinoma SW480 and HT1080 (IC_{50} values of 12.4, 12.2, 21.4 and 8.8 μM, respectively) but was relatively inactive when tested against a panel of breast carcinoma cell lines. The activity did not relate to the presence of mutant or wild-type *ras* in the cell lines tested. In conclusion XR 3054 inhibits ras farnesylation, MAP kinase activation and anchorage-independent growth in NIH 3T3 transformed with v12 H-*ras*. Since the antiproliferative effect of the compound is not related to the *ras* phenotype, XR 3054 may also have effects on other cell signalling mechanisms. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: limonene, perrilyl alcohol, farnesyl transferase
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INTRODUCTION

RAS IS a small guanine nucleotide binding protein that transduces biological information from the cell surface to cytoplasmic components within cells. Mutant *ras* oncogenes are frequently found in human tumours [1–7]. Point mutations in the H-, N- and K-*ras* genes are associated with 20% of all human tumours and in pancreatic and colon cancers this percentage rises to 90% and 50%, respectively. Studies of the role of ras signal transduction have shown that p21 ras is an important membrane-associated protein with GTPase activity. The GTPase activity of the oncogenic forms of p21 ras is lower than the normal forms of the protein and is not affected by GTPase activating proteins [8]. Ras normally acts as a relay for signals between cell surface receptor tyrosine kinases and the cytoplasm, and eventually the nucleus. Several recent reports have indicated that following ligand stimulation and phosphorylation of receptor tyrosine kinases, grb2 and Sos bind the activated receptor bringing the exchange factor into close proximity with p21 ras. In turn, p21 ras recruits raf to the membrane which phosphorylates mitogen activated protein kinase kinase (MAPKK), that in turn phosphorylates

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MAP kinase [9–11]. MAP kinase when activated is rapidly translocated to the nucleus and phosphorylates and activates transcription factors. By this process, ligand stimulation of cells is able to effect cell division and differentiation. This seemingly straight-forward signal pathway is complicated by the fact that ras and ras-related proteins have now been implicated in a variety of signalling pathways which result in a variety of cellular effects. Oncogenic forms of p21 ras have a lower GTPase activity than normal p21 ras and will therefore remain in the activated GTP-bound form for longer, leading to unregulated, sustained signalling through downstream effectors.

To be functional, p21 ras must undergo a post-translational prenylation at its carboxy terminal end. This occurs by attachment of a 15 carbon isoprene unit farnesyl to the cysteine residue in a CAAX motif (C-cysteine, A-aliphatic, X-any amino acid (typically: X = met, ser, gln (FPT); X = leu (GGPT-1)) [12]. This is followed by proteolysis, carboxymethylation and, in the case of N-and H-ras, palmitoylation. Farnesylation occurs in order that p21 ras can be localised at the cell membrane and the process is catalysed by farnesyl protein transferase (FPTase). FPTase transfers farnesyl from farnesyl pyrophosphate to the cysteine of proteins containing the CAAX motif, and it has been the target for a number of groups examining ways of interrupting ras function and signal transduction. A variety of strategies have been developed either using natural products, e.g. manumycin [13] or concentrating on the CAAX motif either by the synthesis of biomimetic compounds such as the benzodiazepines (for review see [14]), tetrapeptide peptidomimetric analogues incorporating novel amino acids. Other approaches include the use of bisubstrate analogues of the farnesyl pyrophosphate group [11–15].

Several compounds have been produced which are capable of inhibiting FPTase at low nanomolar concentrations, but, the lipid solubility of all these inhibitors has been a problem. This problem is now being addressed with modifications to improve the solubility of inhibitor compounds. Limonene and particularly its metabolites has FPTase inhibitory activity and limonene has been shown to cause tumour regression in an animal model of human breast cancer [16, 17]. With the initial observation that limonene causes the regression of methylnitrosourea (MNU)-induced tumours in rats, we have synthesised and screened analogues and closely related compounds of limonene. We report here the cellular and biochemical properties of one of these compounds, XR 3054, in relation to p21 ras function.

MATERIALS AND METHODS

Preparation of XR 3054

Screening of natural and synthetic analogues of limonene was undertaken with a farnesyl transferase [3H]-scintillation proximity assay (SPA) enzyme assay incorporating the scintillation proximity principle (Amersham International, Bucks, U.K.) and validated with brain-derived FTPase. Briefly, a human lamin-B carboxy-terminus sequence peptide (Biotin-YRASNRSCAIM) was [3H]-farnesylated at the cysteine residue when processed by FTPase. The resultant [3H]-farnesyl-(CYS)-biotin lamin-B was captured by a streptavidin-linked SPA bead. Tetrapeptide CVLS was included as a positive control. A further screen based upon the ability of compounds to inhibit the piling morphology of p21 ras transformed NIH 3T3 cells, was undertaken. From these screens, XR 3054 was selected and analysed for the inhibition of post-translational farnesylation in cell lines.

The compound was synthesised in distinct stages as shown in Figure 1.

Preparation of 1S,2S,4S-(4-Isopropenyl-7-oxa-bicyclo[4.1.0]hept-1-yl)-methanol. To a suspension of powdered molecular sieves (4°C, 10 g) in dichloromethane (230 ml) at − 20°C was slowly added, to maintain temperature control, diethyl L-tartrate (810 mg, 39.4 mmol), titanium (IV) isopropoxide (0.94 ml, 3.28 mmol) and 70% tertbutyl hydroperoxide solution (70% in toluene, 28.5 ml, 98.5 mmol). The mixture was allowed to stir at − 20°C for 30 min, before the addition of a precooled solution of S-(-)-perillyl alcohol (10 g, 65.6 mmol) in dichloromethane (60 ml). Stirring was continued for a further 3 h before the mixture was poured into a freshly prepared solution of iron(II) sulphate (14 g) and tartaric acid (4.4 g) in water (44 ml). Rapid stirring was maintained for a further 1 h. The organics were separated and stirred with sodium hydroxide solution (2 N, 100 ml), separated and washed with brine solution (50 ml) and dried over magnesium sulphate. The organics were then filtered and concentrated *in vacuo*. Chromatography on silica gel, eluting with 50% diethyl ether/hexane gave (4-isopropenyl-7-oxa-bicyclo[4.1.0]hept-1-yl)-methanol (7.64g, 68%, 64%de) as a colourless oil.

Preparation of cis-1-hydroxymethyl-4-isopropenyl-cyclohexanol. To a suspension of lithium aluminium hydride (864 mg, 22.7 mmol) in tetrahydrofuran (100 ml) at 0°C, under nitrogen atmosphere, was slowly added a solution of (4-isopropenyl-7-oxa-bicyclo[4.1.0]hept-1-yl)-methanol (7.64 g, 45 mmol) in tetrahydrofuran (20 ml) and the mixture stirred at 0°C for 2 h. The reaction was quenched by the sequential dropwise addition of water (0.9 ml), sodium hydroxide (2 N, 0.9 ml) and water (0.9 ml) and allowed to stir for 1 h. The suspension was filtered and concentrated *in vacuo*. Chromatography on silica gel, eluting with 80% diethyl ether/hexane gave 1-hydroxymethyl-4-isopropenyl-cyclohexanol (6.3 g, 82%) as a white waxy solid.

Preparation of cis-8-isopropenyl-1-2,2-dimethyl-1,3-dioxaspiro [4.5] decane (XR 3054). To a solution of 1-hydroxymethyl-4-isopropenyl-cyclohexanol (6.29 g, 37.0 mmol) in ethylene glycol dimethylether (50 ml) was added para-toluene sulphonic acid (10 mg) and 2,2-dimethoxypropane (5.0 ml,

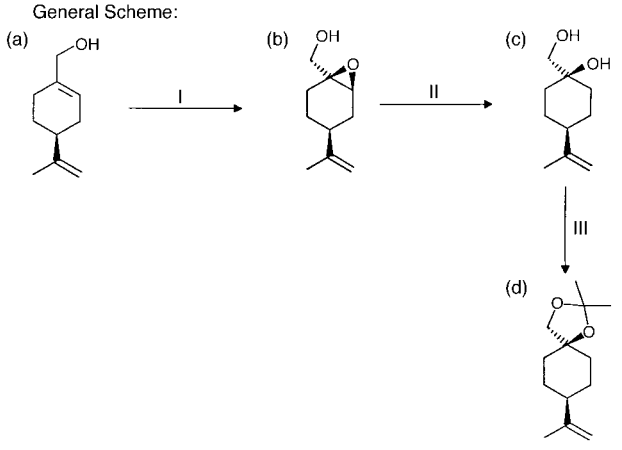

Figure 1. S-(-)-perillyl alcohol (a) was used to prepare 1S,2S,4S-(4-isopropenyl-7-oxa-bicyclo[4.1.0]hept-l-yl) methanol (b). This was converted to (c):cis-l-hydroxy methyl-4-isopropenyl-cyclohexanol and subsequently to (d): cis-8-isopropenyl-2-2-dimethyl-1-3-dioxaspiro[4,5]decane (XR 3054).

40.7 mmol) and the solution stirred at room temperature for 4 h. Sodium carbonate (solid, 200 mg) was added to the mixture and concentrated *in vacuo*. Chromatography on silica gel 1% diethyl ether/hexane gave 8-isopropenyl-2,2-dimethyl-1,3-dioxa-spiro[4.5]decane (4.7 g, 60%) as a colourless oil.

Cell culture and sulphorhodamine red (SRB) cytotoxicity assays

Constitutively active stably transformed NIH 3T3 cells containing oncogenic *ras* and *raf* (V12p21-H-*ras* and V *raf*, respectively) were both gifts from C. Marshall, I.C.R., London, U.K. Two cell lines, HT1080 (fibrosarcoma), containing constitutively active N-*ras* and SW480 (colon carcinoma) (ECACC, Porton Down, U.K.) with a mutated form of K-*ras* were used to monitor ras function and the effect of the compounds. Breast cancer cell lines MDA MB 231 (V12 mutation) (Mason Res. Inst., Rockville, Maryland, U.S.A.) and the following cell lines with wild-type *ras* were also used in experiments. HBr SV161 (M. O'Hare, ICR, U.K.), MDA MB 453, HBL 100, PC3, LnCAP, A431 (all from American Tissue Culture Collection—ATCC, Rockville, Maryland, U.S.A.) were grown in either Dulbecco's modified Eagle's medium (DMEM) or RPMI (Sigma Chemical Co., Poole, Dorset, U.K.) with 10% fetal calf serum and micro-nutrients as previously described [18] were used to evaluate compound XR 3054.

The effects of XR 3054 on various cell lines were tested by a method previously described [19]. Briefly, the assays were carried out using 96-well microtitre plates cells seeded at 2500 cells per well and allowed to establish for 3 days. Compounds were then introduced and incubated for the times indicated in the results. Compounds were replenished daily. Cells were then fixed with 50% ice-cold trichloroacetic acid (TCA) for 1 h, rinsed with water five times and stained with 0.5% sulphorhodamine B for 1 h. Cells were then rinsed in 1% acetic acid and left to dry overnight. Finally plates were read by dissolving the dye in 100 µl of unbuffered 10 mM Tris in a spectrophotometer measuring at 570 nm.

The soft agar assays for the determination of oncogenicity were carried out by inoculating 3×10^3 cells per ml into 0.7% noble agar DMEM 5% fetal calf serum (FCS) (Sigma) with the appropriate concentration of drugs. After 9 and 21 days of incubation colonies were counted.

We also compared the effects of XR 3054 [20] with a previously published inhibitor of farnesyl transferase, SCH 44342, kindly given to us by W.R. Bishop (Schering-Plough, New Jersey, U.S.A.).

MAP kinase assay

NIH 3T3 cells, either parental or transformed with either V12 H-*ras* or v-*raf*, were grown in 9 cm^2 tissue culture plates starved for 24 h in serum-free media, then stimulated with 10 ng/ml epidermal growth factor (EGF) for 5 min. Cell lysates were analysed by SDS-PAGE 12% acrylamide, transferred to nitrocellulose membranes and probed with a polyclonal antibody against ERK2 (E122 B 5-1) (a gift of C. Marshall, ICR, U.K.). Isoprenylation of p21 ras was measured in NIH 3T3 cells transformed V12 H-*ras* as described previously [21]. Briefly, cultures of cells were incubated with 30 uM lovastatin for 24 h and with differing concentrations of the test compounds in serum-deprived media. At the end of this period, the cultures were transferred to media containing 15 uCi/ml of radiolabelled (R,S) -[2-^{14}C]- mevalactone (Amersham). After incubation, the cells were lysed in SDS-PAGE buffer and analysed on 15% PAGE gels. The gels were incubated for 15 min in Amplify (Amersham) fluorographic reagent, dried and analysed with a Bio-Rad GS 250 Molecular Imager.

Analysis of p21 ras mobility shift in the presence of XR 3054

Western blotting for p21 ras was carried out by analysing cell lysates by SDS-PAGE transferred to nitrocellulose membrane and probed with monoclonal antibody Y13-259 (gift from ICR, Sutton, U.K.) and detected with enhanced chemiluminescence (ECL) (Amersham) on X-ray film.

RESULTS

Effect of XR 3054 on ras farnesylation

Positive control tetrapeptide CVLS and the non-peptidyl farnesyl mimetic XR 3054 were found to inhibit farnesylation of the CAAX peptide by FTPase in the SPA assay with IC_{50}s of ~3 µM and ~50 µM, respectively (Figure 2). To confirm that XR 3054 was indeed inhibiting the addition of farnesol to p21 ras, the effect of XR 3054 on the incorporation of ^{14}C-mevalactone into p21 ras was examined. Figure 3a shows there was decreased migration of p21 ras at a concentration of 1×10^{-5} M XR 3054, indicating a reduction of farnesylated ras. The results indicate that XR 3054 inhibits incorporation of ^{14}C-mevalactone into proteins of approximately 21 and 60 and, to a lesser extent, 200 kDa in V12 p21-H-*ras* NIH 3T3 cells (results not shown). Inhibition of ras farnesylation was confirmed using an antibody against the farnesyl motif. The effect of XR 3054 on the MAP kinase ERK2, a downstream effector of p21 ras, was then investigated, analysing the phosphorylation of ERK2 on stimulation with EGF. Figure 3b shows that, at concentrations of XR 3054 known to inhibit the farnesylation of p21 ras in cell culture, there was reduced phosphorylation of ERK2 MAP kinase after 5 min stimulation with EGF (no MAP kinase activation was seen in the absence of EGF). The parental cell line NIH 3T3 and the V12 H-*ras* transformed cell line were both refractile to ligand stimulation, whereas v-*raf* transformed NIH 3T3 cells still showed hyperphosphorylation of MAP kinase on ligand stimulation.

Inhibition of cell proliferation and focus formation using XR 3054

The ability of XR 3054 to inhibit tumour cell proliferation *in vitro* was examined. In these experiments, XR 3054 was first compared with equimolar concentrations of a known

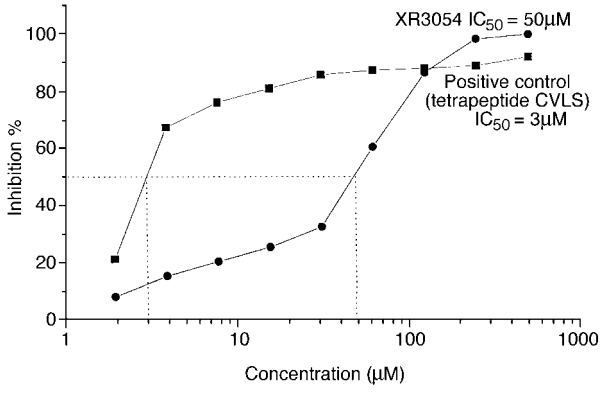

Figure 2. Relative IC_{50} values for the positive control tetrapeptide CVLS and the non-peptidyl synthetic limonene analogue, XR 3054, in the SPA assay.

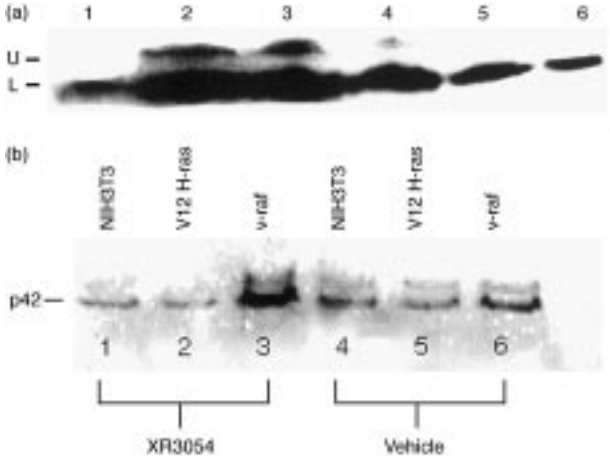

Figure 3. (a) Incorporation of farnesol group into H-*ras* as monitored by SDS-polyacrylamide gel electrophoresis (15% acrylamide). (U) indicates unprocessed (unfarnesylated) H-*ras*; (L) indicates lipidated H-*ras*. Cell lysates, carefully loaded with equivalent amounts of protein, were transferred to nitrocellulose then probed with anti-ras antibody Y13-259 and developed with anti-rat antibody conjugated to horseradish peroxidase (HRPO) and developed with ECL reagents. Lane 1, control V12 H-*ras* transformed NIH 3T3 cells incubated in DMEM media 5% donor calf serum, lanes 2, 3, 4, 5 and 6, the same cell preparation incubated for 36 h with differing concentrations of XR 3054: (2) 5×10^{-4} M; (3) 1×10^{-5} M; (4) 1×10^{-6} M; (5) 1×10^{-7} M; (6) 5×10^{-7} M. (b) The effect of XR 3054 (lanes 1–3) or vehicle (lanes 4–6) on ERK2 phosphorylation in NIH 3T3 parental cells (lanes 1 and 4), H-*ras* transformed cells (lanes 2, 5) and v-*raf* transformed cells (lanes 3, 6). Cells were grown for 3 days and incubated for 36 h in the presence of XR 3054 (5×10^{-5} M), serum deprived for 24 h then stimulated with 10 ng/ml EGF for 5 min. ERK2 phosphorylation was assessed by SDS-PAGE (12% acrylamide) and probed with anti-ERK2 antibody E-122(B5-1) and developed with ECL reagents.

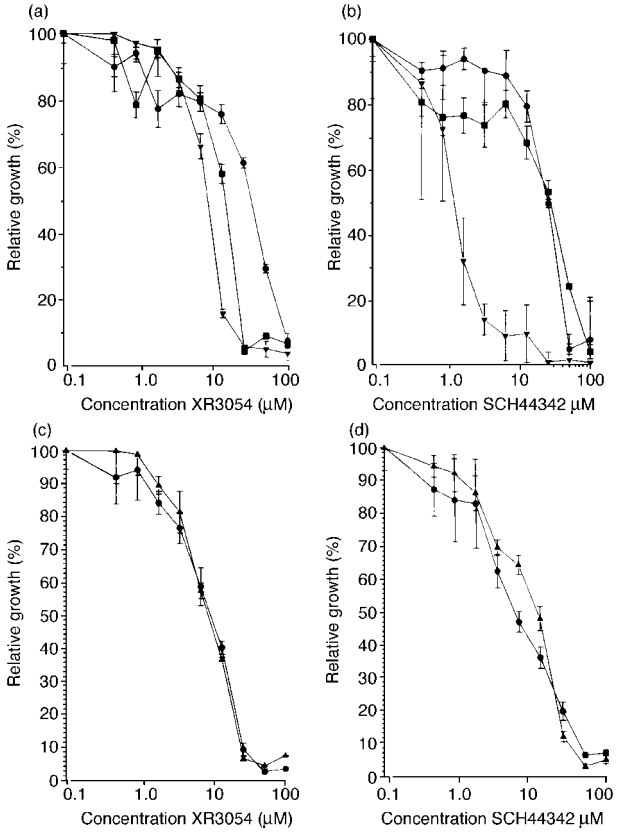

Figure 4. Effect of farnesylation inhibition on the growth of NIH 3T3 cells transformed with H-(▼), K-(■) and N-(●) *ras* (a–b) and on the growth of prostatic cancer cells lines PC3 (●) and LN CAP (▲) (c–d).

tricyclic inhibitor of farnesyl protein transferase SCH 44342 [21] and with carvone, which, like limonene, is a terpene (Table 1). The results indicate that both XR 3054 and SCH 44342 are active against cells with dominant H-, N- and K-*ras* phenotypes and these compounds are broadly equipotent (Table 1, Figure 4). Carvone is less active against NIH 3T3 cells. Table 2 shows that XR 3054 is cytotoxic against a variety of cell lines in which p21 *ras* is the dominant oncogene but is also effective in cell lines in which *ras* is present in its wild-type form, such as the prostatic carcinoma cell lines (IC_{50} of 12.4 and 12.2 μM) (Figure 4).

To determine whether the reduction in farnesylation of p21 ras had an effect on the transforming capability of V12 H-ras the ability of cells to form foci in soft agar in the presence of XR 3054 was tested. Treatment with XR 3054 resulted in an inhibition of the number of colonies in soft agar with an IC_{50} value of 3×10^{-5} M. Transformed NIH 3T3 cells with constitutively active v-*raf* were unaffected by the presence of XR 3054 (Figure 5). These data indicate that XR 3054 affects the growth of NIH 3T3 at the level of p21 ras or above in the signal cascade.

Table 1. *Effect of farnesyl transferase inhibitors on proliferation of cell lines (IC_{50} shown)*

Cell line Ras phenotype	NIH 3T3 H-*ras*	HT1080 N-*ras*	MCF7 wt	SW480 K-*ras*
Compound				
SP44342 (μM)	36.4	2.24	11	39.6
XR 3054	18	8.8	58	21.4
Carvone (μM)	87.2	–	–	–

DISCUSSION

In this study a limonene analogue XR 3054 was investigated and shown to inhibit p21 ras farnesylation *in vitro*. The screening of limonene analogues in an *in vitro* FPTase assay

Table 2. *Inhibitory activity of XR* 3054 *against a variety of cell lines*

Cell line (μM)	type	*Ras*	IC_{50}
LnCAP	prostate	wt	12.4
PC3	prostate	wt	12.2
A431	vulval	wt	> 500
SW480	colon	(K V12)	21.4
NIH 3T3	fibroblast	(H V12)	18.0
HR1080	colon	wt(N)	8.8
MDA MB 231	breast	V12	198
MDA MB 157	breast	wt	> 500
MDA MB 453	breast	wt	213
HBL100	breast	wt	154
Hbr	breast	wt	> 97

Cells were seeded and, at day 3 of incubation, were treated with XR 3054 at a variety of concentrations for 5 days and assayed as described in the text.

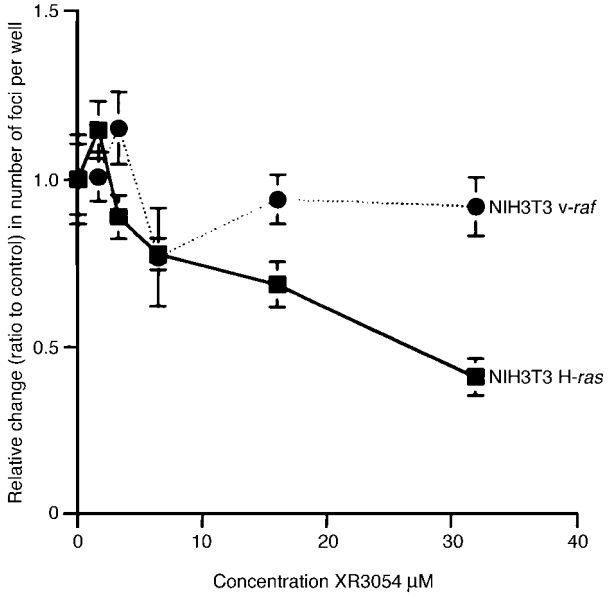

Figure 5. Effect of XR3054 on the growth of H-*ras* and v-*raf* transformed NIH3T3 cells in soft agar, as described in the Methods.

showed that XR3054 was effective in inhibiting FPTase in the micromolar range. Data from a cell-based screen in which candidate compounds were screened for their ability to reverse the cell-piling phenotype of p21 *ras* transformed cells in cell culture indicated that XR3054 was effective at reversing this phenotype. The *in vitro* cytotoxicity experiments showed that XR3054 was able to inhibit the growth of cell lines in the micromolar range and the compound is of comparable activity to SCH 44342 [18], although this compound has a much more potent activity in blocking ras processing ($IC_{50} = 1\,\mu M$). The cytotoxic effect of XR3054 occurs at lower concentrations than the *in vitro* screen of FPTase would suggest. From this it could be concluded that XR3054 might not be acting against the FPTase alone. Initial studies carried out by our group failed to disclose any potent inhibition of GGTase (D. Vigushin, Imperial College, London, U.K.) but further studies are required to determine whether XR3054 inhibits Type 1 or Type 2 GGTases. Perillyl alcohol has been reported to inhibit GGTase in NIH3T3 cells [22].

It was also found that EGF-stimulated MAP kinase activation in quiescent NIH3T3 cells was reduced in the presence of XR3054 at concentrations known to reduce ras farnesylation, and this appears similar to the effects observed using a peptidomimetic inhibitor of FPTase [23].

XR3054 also inhibited anchorage-independent growth of a H-*ras* transformed cell line at 30 μM while not affecting the anchorage-independent growth of v-*raf* transformed cells. In this respect XR3054 is slightly more potent when compared with other reported FPTase inhibitors where the IC_{50} values range from 50 to 100 μM [15, 24, 25]. The inhibition of anchorage-independent growth and inhibition of monolayer growth is thought to be appropriate for examining ras transforming ability in p21 *ras* transformed cells. However, evidence from a wider range of cell types in which p21 *ras* is not implicated as the main oncogene suggests that the XR3054 is not acting solely as an inhibitor of ras farnesylation. The data presented here indicate that XR3054 is capable of inhibiting cell proliferation in cell lines bearing activating p21 *ras* mutations, but there are striking examples of cell lines which do not bear p21 *ras* mutations which are sensitive to XR3054, e.g. LnCAP, PC3.

Limonene-like compounds may have multiple mechanisms of action in mammalian cells. This is suggested not only by this study but also by the results of our phase 1 study of limonene in patients with cancer in which patients tolerated up to $8\,g/m^2$ oral limonene [26]. We have previously found that regression of breast carcinoma could occur with limonene at dose levels less than would be expected to inhibit ras farnesylation, and postulated that this could be due to the metabolism of limonene to several active metabolites including perillic acid and dihydroperillic acid [27]. *In vivo* metabolism of XR3054 has not yet been studied but, like limonene, it is possible that active metabolites may be formed which could contribute to its antitumour activity.

Limonene and other monoterpenes may also possess other mechanisms of action such as an enhancement of both the production and activation of the potent growth inhibitor TGFβ [28]. In addition, mammary tumours regressing after D-limonene showed an upregulation of mRNA for the M6P/IGF II receptor which binds the TGFβ latent complex, which can result in plasmin-induced extracellular TGFβ activation [29, 30]. It is not yet known which of these mechanisms is responsible for XR3054 action, nor whether XR3054 treatment results in apoptosis, such as is seen after perillyl alcohol treatment in animals bearing liver tumours [31].

Whatever the mechanism of action, limonene and its derivatives and metabolites remain attractive candidates as antitumour compounds which may be potential chemopreventative agents in view of their tolerability at high doses.

1. Barbacid M. Ras genes. *Ann Rev Biochem* 1987, **56**, 779–827.
2. Grand RJA, Owen D. The biochemistry of ras p21. *Biochem J* 1991, **279**, 609–631.
3. Barbacid M. Human oncogenes 1986. In Devita VT, Hellman S and Rosenberg S. eds. *Important Advances in Oncology*. Philadelphia, J.B. Lippincott, 3–22.
4. Mulcahy LS, Smith MR, Stacey DW. Requirement for ras proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. *Nature* 1985, **313**, 241–243.
5. Noda M, Ko M, Ogura A, *et al.* Sarcoma viruses carrying ras oncogenes induce differentiation-associated properties in a neuronal cell line. *Nature* 1985, **318**, 73–75.
6. Bar-Sagi D, Feramisco JR. Microinjection of ras oncogene protein into PC12 cells induces morphological differentiation. *Cell* 1985, **42**, 841–848.
7. Kataoka T, Powers S, McGill C, Fasano O, Strathern J, Wigler M. Genetic analysis of yeast Ras1 and Ras2 genes. *Cell* 1984, **37**, 437–445.
8. McCormick F. The world according to GAP. *Oncogene* 1990, **5**, 1281–1283.
9. Leevers SJ, Paterson HF, Marshall CJ. Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. *Nature* 1994, **369**(6479), 411–414.
10. Marshall CJ. MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr Opin Genet Dev* 1994, **4**(1), 82–89.
11. Troppmair J, Bruder JT, Munoz H, *et al.* Mitogen-activated protein kinase/extracellular signal-regulated protein kinase activation by oncogenes, serum and 12-0-tetradecanoylphorbol-13-acetate requires Raf and is necessary for transformation. *J Biol Chem* 1994, **269**, 7030–7035.
12. Hancock JF, Magee AI, Childs JE, Marshall CJ. All ras proteins are polyisoprenylated but only some are palmitoylated. *Cell* 1989, **57**, 1167–1171.
13. Hara H, Han M. Ras farnesyltransferase inhibitors suppress the

phenotype resulting from an activated ras mutation in *Caenorhabditis elegans*. *Proc Natl Acad Sci USA* 1995, **92**, 3333–3337.
14. Gibbs JB, Oliff A, Kohl NE. Farnesyltransferase inhibitors: ras research yields a potential cancer therapeutic. *Cell* 1994, **77**, 175–178.
15. Manne V, Yan N, Carboni JM, *et al*. Bisubstrate inhibitors of farnesyltransferase: a novel class of specific inhibitors of ras transformed cells. *Oncogene* 1995, **10**, 1763–1779.
16. Elegbede JA, Elson CE, Qureshi A, Tanner MA, Gould MN. Inhibition of DMBA-induced mammary cancer by the monoterpene D-limonene. *Carcinogenesis* 1998, **5**, 661–664.
17. Chander SK, Lansdowne AGB, Luqmani YA, *et al*. Effectiveness of combined limonene and 4-hydroxyandrostenedione in the treatment of NMU-induced rat mammary tumours. *Br J Cancer* 1994, **69**, 879–882.
18. Luqmani YA, Graham M, Coombes RC. Expression of basic fibroblast growth factor, FGFR1 and FGFR2 in normal and malignant human breast, and comparison with other normal tissues. *Br J Cancer* 1992, **66**, 273–280.
19. Skehan R. New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 1990, **82**, 1107–1112.
20. Bishop WR, Bond R, Petrin J, *et al*. Novel tricyclic inhibitors of farnesyl protein transferase. Biochemical characterisation and inhibition of Ras modification in transfected Cos cells. *J Biol Chem* 1995, **270**, 30611–30618.
21. Leftheris K, Kline T, Vite GD, *et al*. Development of highly potent inhibitors of Ras farnesyltransferase possessing cellular and in vivo activity. *J Med Chem* 1996, **39**(1), 224–236.
22. Ren Z, Elson CE, Gould MN. Inhibition of Type I and Type II geranylgeranyl-protein transferases in the monoterpene perillyl alcohol in HIH3T3 cells. *Biochem Pharmacol* 1997, **54**, 113–120.
23. Sepp-Lorenzino L, Ma Z, Rands E, *et al*. A peptidomimetic inhibitor of farnesyl:protein transferase blocks the anchorage-dependent and -independent growth of human tumor cell lines. *Cancer Res* 1995, **55**, 5302–5309.
24. James GL, Goldstein JL, Brown MS, *et al*. Benzodiazepine peptidomimetics: potent inhibitors of ras farnesylation in animal cells. *Science* 1993, **260**, 1937–1942.
25. Nagase T, Kawata S, Tamura S, *et al*. Inhibition of cell growth of human hepatoma cell lines (Hep G2) by a farnesyl protein transferase inhibitor: a preferential suppression of ras farnesylation. *Int J Cancer* 1996, **65**, 620–626.
26. Vigushin DM, Poon GK, Boddy A, *et al*. Phase I and pharmacokinetic study of D-limonene in patients with advanced cancer. *Cancer Chemother Pharmacol* 1998, **42**, 111–117.
27. Poon GK, Vigushin D, Griggs LJ, Rowlands MG, Coombes RC, Jarman M. Identification and characterisation of limonene metabolites in patients with advanced cancer by liquid chromatography/mass spectometry. *Drug Metab Disposition* 1996, **24**, 565–571.
28. Jirtle RL, Carr BI, Scott. CD. Modulation of insulin-like growth factor-II/mannose 6-phosphate receptors and transforming growth factor-beta 1 during liver regeneration. *J Biol Chem* 1991, **266**, 22444–22450.
29. Kornfeld S. Structure and function of the mannose 6-phosphate/insulin-like growth factor II receptors. *Annu Rev Biochem* 1992, **61**, 307–330.
30. Dennis PA, Rifkin DB. Cellular activation if latent transforming growth factor-β requires binding to the cation-independent mannose 6-phosphate/insulin-like growth factor type II receptor. *Proc Natl Acad Sci USA* 1991, **88**, 580–584.
31. Mills JJ, Chari RS, Boyer IJ, Gould MN, Jirtle RL. Induction of apoptosis in liver tumors by the monoterpene perillyl alcohol. *Cancer Res* 1995, **55**, 979–983.

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