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Inhibitors of Prenylation of Ras and Other G-proteins and Their Application as Therapeutics

Louis H. Cohen,† Elsbet Pieterman,* Rick E. W. van Leeuwen,* Mark Overhand,‡ Brigitte E. A. Burm,‡ Gijs A. van der Marel‡ and Jacques H. van Boom‡*

*Gaubius Laboratory, TNO Prevention and Health and ‡Department of Organic Chemistry, Leiden University, Leiden, The Netherlands

ABSTRACT. Anchoring of small G-proteins to cellular membranes via a covalently bound lipophylic prenyl group is essential for the functioning of these proteins. For example, the farnesylation of Ras by the action of the enzyme protein:farnesyl transferase (PFT) is pivotal for its signalling function in cell growth and differentiation. The development of inhibitors of PFT was triggered by the role of mutated Ras in certain types of cancer and by the observation that non-farnesylated Ras is inactive. Besides the screening of existing compounds for PFT inhibition, rational drug design has also led to new inhibitors. Our research is in the field of atherosclerosis and concerns the development of inhibitors of the growth of vascular smooth muscle cells. The latter process gives rise to reocclusion of the coronary artery (restenosis) after balloon angioplasty. We and others have developed several analogues of the two substrates of PFT, i.e. farnesyl pyrophosphate (FPP) and the so-called CAAX peptide consensus sequence, which were tested *in vitro* for the inhibition of PFT and of other enzymes involved in protein prenylation, such as protein:geranylgeranyl transferase-1 (PGGT-1). The FPP analogue TR006, a strong inhibitor of PFT (IC_{50} of 67 nM), blocked the proliferation of cultured human smooth muscle cells and inhibited platelet-derived growth factor- and basic fibroblast growth factor-induced DNA synthesis. Similar but more highly charged compounds failed in this respect, probably because of an impaired uptake in the cells. Less charged derivatives were designed to circumvent this problem. The effect on the GF-induced activation of intermediates in signal transduction pathways was investigated in order to gain insight into the mechanism of action within the cells. TR006 decreased the bFGF activation of extracellular signal-regulated kinase 1 (ERK1), suggesting its involvement in inhibiting Ras activity. Although other analogues inhibited DNA synthesis, they affected neither ERK1 activation nor p38/stress-activated protein kinase 2 or Jun N-terminal kinase 1 activation. Since some of these compounds were also shown to be inhibitors of *in vitro* PGGT-1 activity, the geranylgeranylation of other G-proteins may be decreased by these compounds. Rho seems to be a good candidate as a target for inhibitors of PGGT-1. This uncertainty as to the mechanism of action within non-transformed as well as transformed cells applies to all prenylation inhibitors, but is not holding back their further development as drugs. Their current and possible future application as therapeutics in cancer, restenosis, angiogenesis, and osteoporosis is briefly discussed. BIOCHEM PHARMACOL **60**;8:1061–1068, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. protein:farnesyl transferase; protein:geranylgeranyl transferase; human smooth muscle cells; restenosis; cancer; angiogenesis; osteoporosis

Small G-proteins that play a role in signal transduction must be anchored via a posttranslationally added lipophylic (iso)prenyl group to cellular membranes to be able to perform their function. As shown in Fig. 1, the prenyl groups (e.g. farnesyl in Ras and geranylgeranyl in Rac and Rho) are derived from FPP§, an intermediate of the cholesterol biosynthesis pathway, and are covalently bound to the above-mentioned G-proteins by the enzymes PFT and PGGT-1. The research in the field of protein (iso)prenylation has increased rapidly during the last decade and has been reviewed extensively [1–4]. It all started with the observation that inhibitors of the enzyme HMG-CoA reductase (a group of compounds designated as statins and currently in use as cholesterol-lowering drugs) influence proliferation, differentiation, and migration of cells in culture. This action was associated with the prevention of prenylation of G-proteins [5]. More recently, the same activity was observed for a group of anti-osteoporosis drugs, the NCBPs [6, 7], which is attributed to their recently discovered action as specific inhibitors of the enzyme FPP synthase (Fig. 1) [8–10].

The notions that Ras must be farnesylated for it to be active in growth factor-activated signal transduction and that ras mutations are strongly associated with the occur-

† Corresponding author: Dr. Louis H. Cohen, Gaubius Laboratory, TNO Prevention and Health, P.O. Box 2215, 2301 CE Leiden, The Netherlands. Tel. +31-71-518.1469; FAX +31-71-518.1904; E-mail: L.H.Cohen@pg.tno.nl

§ *Abbreviations:* PFT, protein:farnesyl transferase; PGGT, protein:geranylgeranyl transferase; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; NCBP, nitrogen-containing bisphosphonate; POM, pivaloyl-oxo-methyl; (H)SMC, (human) smooth muscle cell; HMG, 3-hydroxy-3-methylglutaryl; ERK1, extracellular signal-regulated kinase 1; SAPK2, stress-activated protein kinase 2; and JNK1, Jun N-terminal kinase 1.

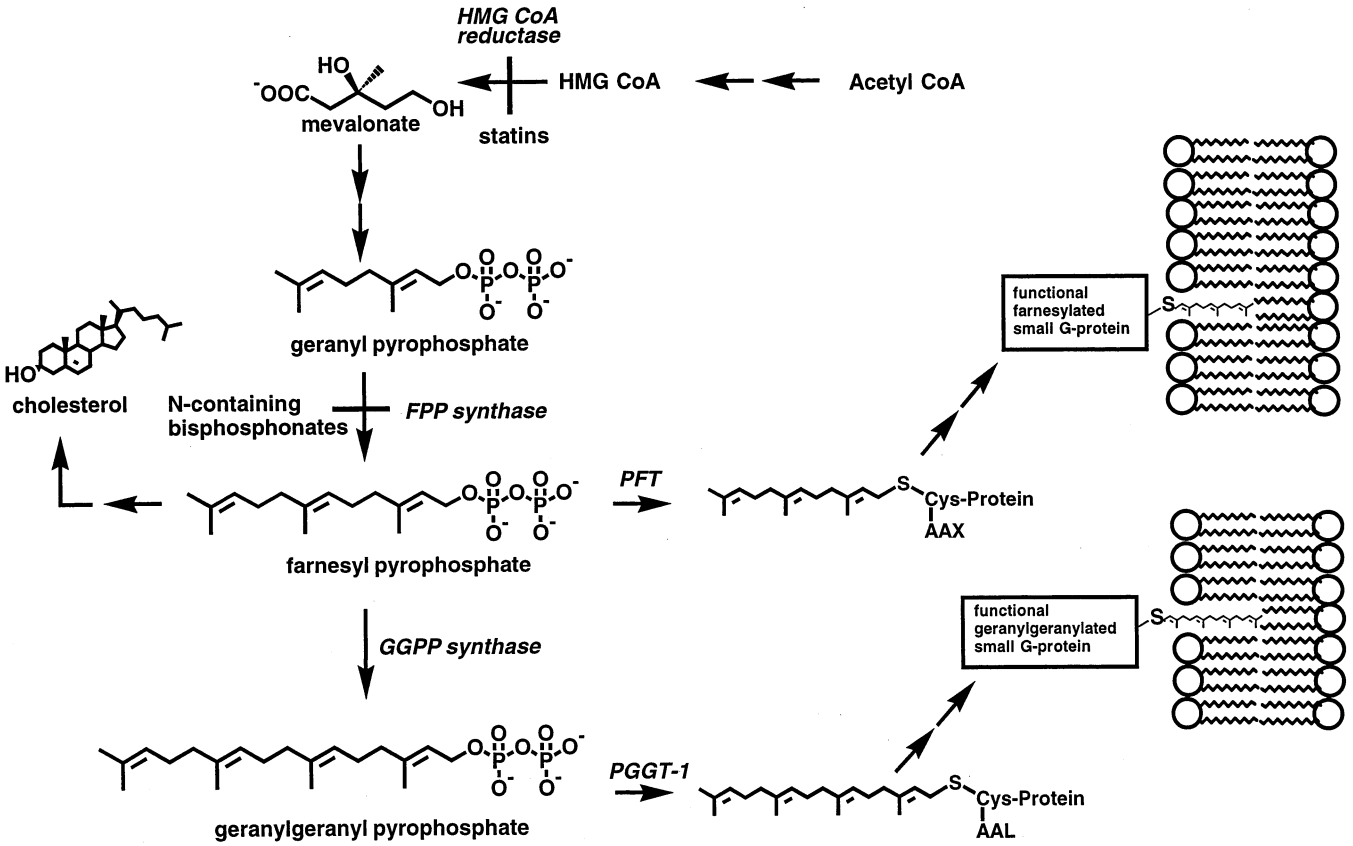

FIG. 1. Biosynthesis pathway of isoprenoids leading to prenylated proteins. Statins are specific inhibitors of HMG-CoA reductase (the rate-limiting enzyme of cholesterol biosynthesis) and NCBPs of the enzyme FPP synthase. The chemical structures of the prenyl groups are shown and the positions of the protein prenylating enzymes PFT and PGGT-1 are indicated. After prenylation, the proteins are further modified and will finally move to the cellular membranes where they exert their signalling function.

rence of colon and pancreas carcinomas provided the strongest impetus for the search for new anticancer drugs [11–14]. In this case, PFT was the target enzyme of choice. On the other hand, unwanted cell growth also plays a role in the process of atherosclerosis and more evidently in the process of restenosis. Proliferation of SMC takes part in the development of atherosclerotic plaques in the artery wall and is the major cause of reocclusion of the coronary artery (restenosis) after balloon angioplasty [15]. Thus, inhibition of this cell growth will be beneficial. Although ras activation is important for the proliferation of these cells [16], other G-proteins may also be involved (see below) and consequently, in addition to PFT, PGGT-1 should be a target enzyme for the development of inhibitors of SMC proliferation. While protein prenylation is associated with cell growth, the potential of prenylation inhibitors as therapeutics in various diseases is obvious. Our compounds have been developed for a possible application to prevent restenosis and the examples given below are directed towards this goal. Finally, the current and possible future application of prenylation inhibitors as therapeutics in the treatment of cancer, angiogenesis, and osteoporosis will be briefly discussed.

INHIBITORS OF PFT/PGGT-1

In the search for specific inhibitors of PFT and PGGT-1, two approaches have been used: the screening of existing compound libraries for inhibitory activity and rational drug design based on the characteristics of these enzymes. In accordance with the second approach, we [17–20] and others (reviewed in [12]) have developed several analogues of the two substrates of the enzymes mentioned. For PFT, these are analogues of FPP and mimetics of the carboxy-terminal tetrapeptide of the unprocessed G-protein, which is designated as the CAAX box, in which C = cysteine (to which the farnesyl group is bound), A = an aliphatic residue, and X = methionine or serine. In a slightly different way, analogues of GGPP and peptidomimetics of the CAAL-terminal sequence (the consensus sequence of precursors of several geranylgeranylated proteins, where L stands for leucine) have been designed as inhibitors of PGGT-1 [19, 21, 22]. Additionally, bisubstrate analogues, consisting of both chemical structure elements, have also been developed [23, 24]. Examples of the structures of these inhibitors can be found in several review papers as mentioned above, but a few examples of recently published CAAX mimetics and FPP analogues together with some

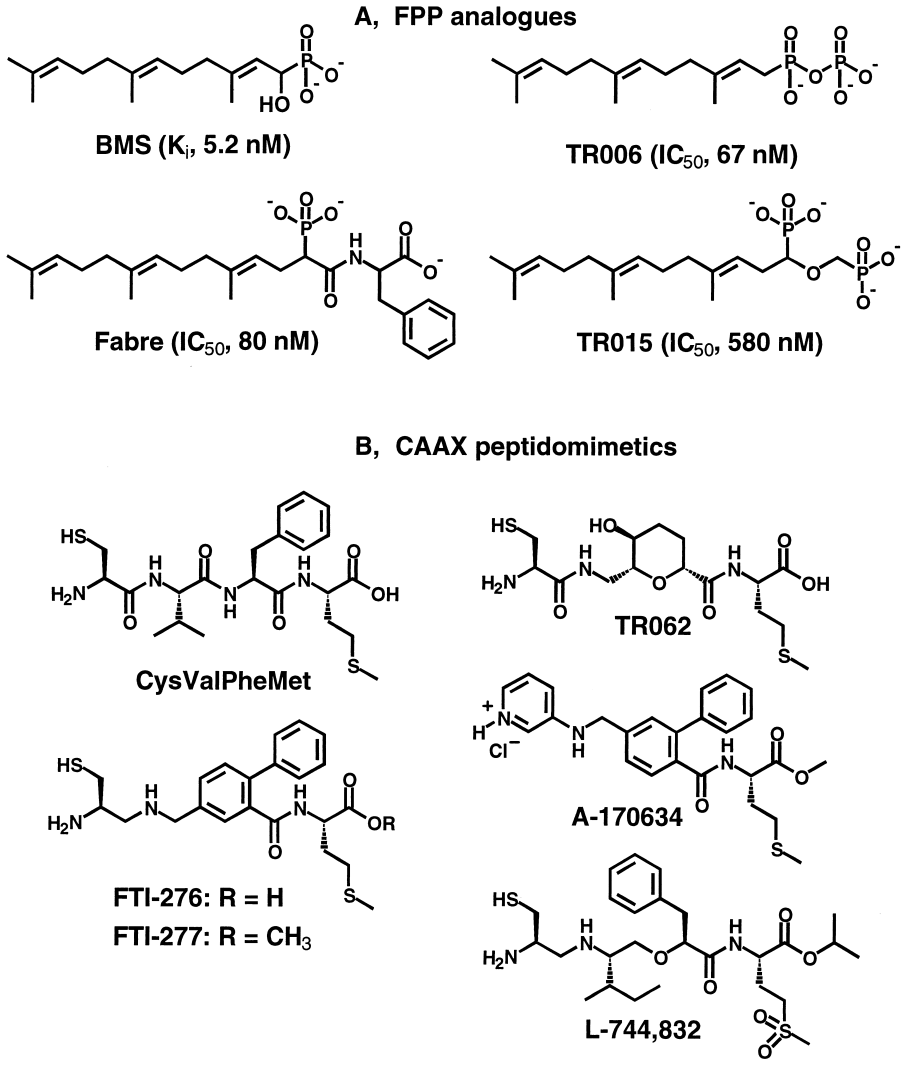

FIG. 2. The chemical structure of some PFT inhibitors ((A) FPP analogues and (B) CAAX peptidomimetics) is given. (A) The indicated IC_{50} values of the FPP analogues were obtained in an *in vitro* enzyme PFT assay. The incubation conditions of this assay were not the same in all cases. Therefore, the IC_{50} values must be considered as an indication, but cannot be used for comparison in an absolute sense. BMS stands for Bristol Myers Squibb. (B) The CAAX tetrapeptide Cys-Val-Phe-Met, which is a PFT inhibitor by itself [58], is depicted for structural comparison with the peptidomimetics shown.

designed by our research group (designated as TR compounds) are depicted in Fig. 2. One of the concerns in developing these compounds is the high polarity of the original substrates and the vulnerability of the peptide analogues to protease degradation. As can be seen in Fig. 2B, there is a tendency to remove peptide bonds and replace aminoacid residues by more lipophylic groups. The presence of many negatively charged phosphate groups in some of the FPP analogues will probably impair cellular uptake, and for that reason compounds have been made in which the charge has been masked by covalently bound POM groups [25]. The POM group is thought to be removed by endogenous hydrolases after entering the cell, and thus the "prodrug" will be converted to the original inhibitor within the cell [26]. An example of such a compound, TR020, is shown in Fig. 3.

ATHEROSCLEROSIS/RESTENOSIS

Our research lies mainly in the field of atherosclerosis and this study in particular concerns the development of inhibitors of the growth of vascular SMC. As expected, statins inhibited the proliferation of SMC in culture and concomitantly decreased the farnesylation of Ras [27]. Furthermore, it was shown that inhibition of Ras activity by transfection with DNA that expressed transdominant negative mutants of Ras led to the inhibition of SMC proliferation in an animal study [16]. For that reason, inhibitors of ras farnesylation should be effective as inhibitors of SMC growth. We tested the FPP analogue TR006 (see Fig. 2), a strong *in vitro* inhibitor of PFT, in a series of cellular studies. Previously, it was shown that this compound inhibited the farnesylation of Ras in a Ha-ras-transfected cell line [28] and concomitantly slowed down the growth of these cells

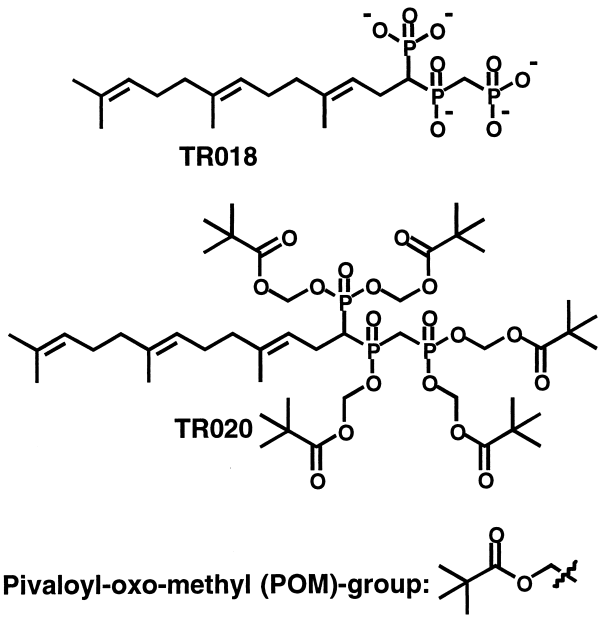

FIG. 3. POM derivative of an FPP analogue. In compound TR020, the highly negative charge (−5) of TR018 has been masked by addition of POM groups to the charged oxygen atoms. The POM group is thought to be removed by endogenous hydrolases after entering the cell, and thus the "prodrug" will be converted to the original inhibitor within the cell.

[29]. The same compound inhibited, in a concentration-dependent manner, the proliferation of cultured SMC, isolated from human left internal mammary artery [29]. In synchronised HSMC, TR006 inhibited PDGF- and bFGF-induced DNA synthesis [29], illustrating that this compound is active in the G1 phase of the cell cycle. This is to be expected if growth factor-stimulated signal transduction through ras activation is affected. Other FPP analogues with comparable IC_{50} values in the PFT assay showed an inhibitory effect on bFGF-stimulated DNA synthesis in HSMC as well [29]. However, more highly charged compounds failed in this respect, probably because of an impaired uptake in the cells. On the other hand, the POM derivatives of the latter compounds, designed to be converted to the original inhibitors within the cell, did inhibit DNA synthesis in synchronised HSMC. Examples are given in Fig. 4A. TR017 and TR020 (Fig. 3), which are the POM derivatives of TR015 (Fig. 2) and TR018 (Fig. 3), respectively, were much stronger inhibitors than TR015 and TR018. The negative charge of diphosphonate compounds such as TR015 (Fig. 2) has been decreased by other methods as well. In this particular case, cyclic compounds were synthesised by connecting the two phosphate groups, thereby forming a pyrophosphate structure with two negative charges less [19]. It is to be expected that an endogenous phosphatase will hydrolyse this bond. In Fig. 4A, an example of the latter compounds has been given as well. TR031 is the cyclic counterpart of TR025, which is an inhibitor of PFT (IC_{50}: 5.3 μM) as well as of PGGT-1 (IC_{50}: 0.98 μM). The cyclic compounds were less active in comparison with their non-cyclic counterparts in the *in vitro* enzyme assays [19], but TR031 was a stronger inhibitor of bFGF-induced DNA synthesis than TR025.

In order to gain more insight into the mechanism of action of these compounds in HSMC, their effect on the bFGF-induced activation of intermediates of several signal transduction pathways, i.e. ERK1, p38/SAPK2, and JNK1, was investigated. As shown in Fig. 4B, TR006 decreased the bFGF-induced activation of ERK1, suggesting the inhibition of Ras activity, but also decreased the activation of p38/SAPK2 and JNK1. Although other FPP analogues, such as TR017, TR020, and TR031, inhibited DNA synthesis, they affected neither bFGF-induced ERK1 nor p38/SAPK2 or JNK1 activation. Since the *in vitro* active counterparts of these compounds (TR015, TR018, and TR025, respectively) were also shown to be inhibitors of PGGT-1 activity [19, 29], the geranylgeranylation of other G-proteins may be decreased by these compounds. Even the PFT inhibitor TR006 may be active through indirectly decreasing the geranylgeranylation of G-proteins, because it also inhibits the activity of the enzyme GGPP synthase (see Fig. 1) *in vitro* (IC_{50}: 2.3 μM), and its inhibition of DNA synthesis in HSMC is counteracted by the co-addition of GGPP.* Moreover, TR006 decreased the incorporation of radiolabelled mevalonate into geranylgeranylated proteins in H-ras.13 cells [28]. Other investigations support the importance of geranylgeranylation over that of farnesylation in non-transformed cells. An example thereof is the inhibition of SMC growth by statins, which can be prevented totally by the co-addition of GGPP, but only partly by FPP [30, 31]. In addition, a specific inhibitor of protein:geranylgeranyl transferase, GGTI-298, blocked the growth of pulmonary vascular SMC, but a specific PFT inhibitor did not [32]. Cell growth arrest took place in the transition from G1 to S phase and was associated with the inactivation of Rho G-proteins [30, 31, 33], which normally are geranylgeranylated. It was previously shown that Rho and Rac proteins (the latter are geranylgeranylated as well) play an important role in this cell cycle transition [34]. Although these data suggest that inhibitors of PGGT-1 alone can be developed further into drugs to prevent detrimental proliferation of non-transformed cells, it cannot be excluded that farnesylation is affected by these compounds as well. Moreover, it is conceivable that both farnesylation and geranylgeranylation must be suppressed to obtain effective inhibition of the proliferation of these cells. With this in mind, it may be sensible to develop inhibitors which are less discriminative between PFT and PGGT-1, such as the compounds described above.

CANCER/ANGIOGENESIS

Since about 30% of all human cancers express mutated Ras proteins [35] and farnesylation of Ras was shown to be

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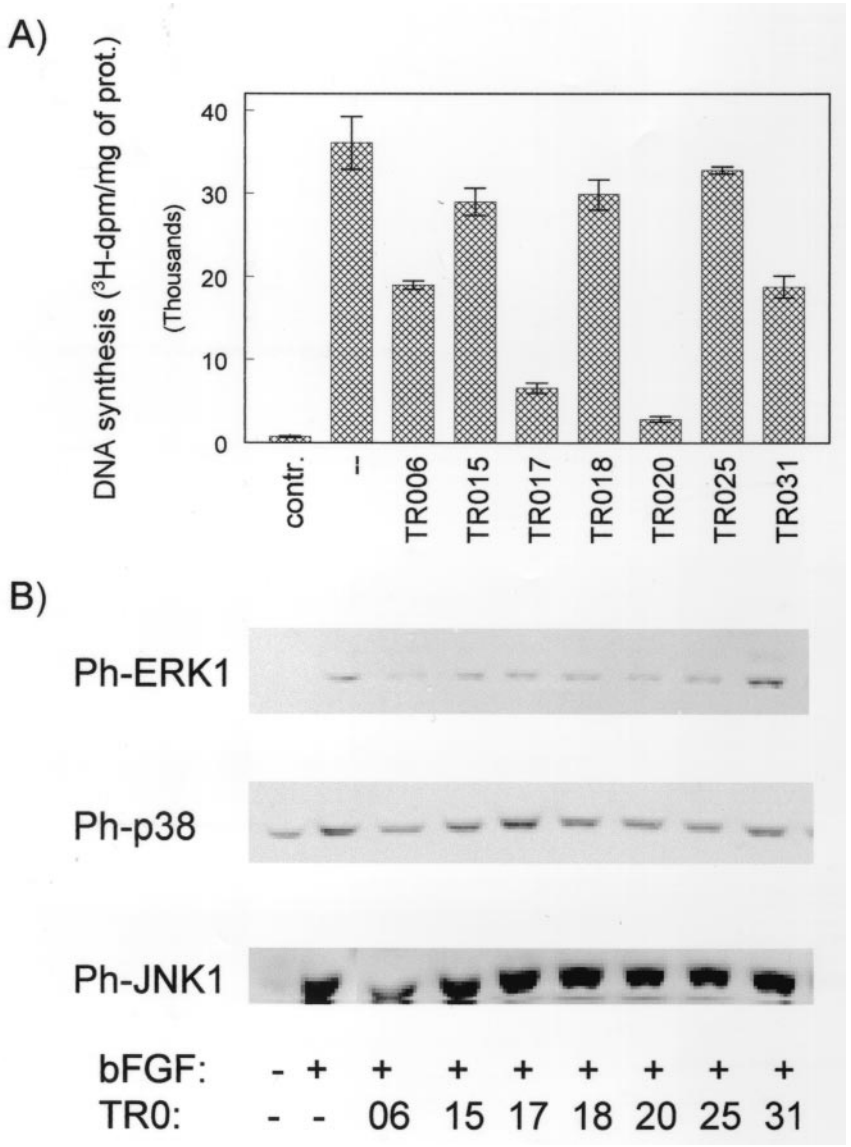

FIG. 4. Effects of FPP analogues on bFGF-induced (A) DNA synthesis and (B) activation of signal transduction intermediates in human smooth muscle cells. As described previously [27, 29], HSMC were isolated from left internal mammary artery, cultured, and synchronised, and their cell cycle was started by addition of bFGF. (A) The indicated FPP analogues (10 μM; contr. = without bFGF) were added at 2 hr before bFGF addition and 27 hr thereafter DNA synthesis (incorporation of radio-labelled uridine into DNA) was measured. (B) bFGF-induced activation of signalling intermediates was detected by Western blot analysis using specific antibodies for the phosphorylated (activated) form of ERK1, p38/SAPK2, and JNK1, indicated as Ph-ERK1, Ph-p38 and Ph-JNK1, respectively.

essential for its function [36, 37], PFT was early on identified as a target for antitumour drugs [38–40]. For this reason, the development of PFT inhibitors as drugs in this field has been rather advanced [12], and some of the selected and/or designed compounds, such as R115777 [41], L-744,832 [42], and SCH 66336 [43], have been tested in phase I clinical investigations. Very recently, the development and application of PFT inhibitors as anticancer drugs was extensively discussed by Rowinsky *et al.* [44]. Notwithstanding the promising results so far, the efficacy of the PFT inhibitors in growth arrest varies among different ras-mutated tumours, and therefore the mechanism of action is not completely understood. On the one hand, this may depend on the different types of the mutated Ras (in the human there are three types: N-ras, H-ras, and Ki-ras), which may behave differently in reaction with PFT and the inhibitor thereof. Moreover, Ki-ras, which is the most frequently mutated form in human cancers, can also be geranylgeranylated and therefore may still be active in the presence of a PFT inhibitor. Indeed, a combination of PFT and PGGT-1 inhibitors blocked the growth of Ki-ras-transformed cells more efficiently than did any of the inhibitors alone [45]. On the other hand, the mutated Ras may not be the primary or the only cause of tumour growth and another (not-yet-identified) farnesylated or geranylgeranylated (G-)protein with a different sensitivity towards prenylation inhibition may be involved. Furthermore, it was shown that malignant cells that lacked a ras

mutation were still sensitive to PFT inhibitors and that Rho was involved [46]. For instance, such an inhibitor caused an accumulation of geranylgeranylated RhoB, which in turn inhibited cell growth [47]. It has to be experienced in practice whether these uncertainties as to the mechanism of action will have an impact on the choice of compound or combination of compounds used to treat different tumours.

The growth of a solid tumour is very much dependent on the ingrowth of new blood vessels for the supply of nutrients [48]. Migration and proliferation of vascular endothelial cells play a major role in this process of angiogenesis. PFT inhibitors, e.g. A-170634 [49] and L-744,832 [50], have been shown to have *in vitro* and *in vivo* anti-angiogenic effects. Again, it is not clear whether the mechanism of action involved inactivation of ras. Our compound TR006 also inhibited microvascular endothelial cell migration in an *in vitro* angiogenesis model [51], but this effect was counteracted by the co-addition of GGPP.* Notwithstanding the uncertainty about the mechanism of action of PFT inhibitors, it is thought to be favourable that these compounds can interfere with tumour growth via multiple effects, i.e. blocking ingrowth of endothelial cells and the proliferation of the tumour cells.

OSTEOPOROSIS/BONE CANCER

A new therapeutic development in the field of osteoporosis will emerge from the discovery of the target enzyme of the NCBPs, which are currently in use as drugs to block osteoclast-mediated bone resorption. We [8, 52] and others [9, 10] have shown that these compounds are strong and specific inhibitors of farnesyl pyrophosphate synthase, an enzyme essential for both cholesterol synthesis and protein prenylation (Fig. 1). Additionally, it was shown that the action of NCBPs on osteoclasts could be prevented by geranylgeraniol but not by FPP [7, 53], suggesting that geranylgeranylation of proteins is necessary for osteoclast-mediated bone resorption. Thus, it is likely that inhibitors of protein prenylation (inhibitors of geranylgeranylation seem a logical choice, although PFT inhibitors are not excluded because of the uncertainty of their mechanism of action within cells), which are preferentially targeted to bone, may be a more specific anti-osteoporosis agent than the NCBPs. Moreover, because osteolysis during bone tumour growth is mediated by osteoclast action as well [54], bone-targeted prenylation inhibitors may do both: inhibition of tumour growth and inhibition of osteoclast-mediated bone resorption induced by the bone tumour.

In contrast to the action of osteoclasts, osteoblasts are the cells that provide new bone formation. Mundy *et al.* recently showed [55] that inhibition of mevalonate production by statins stimulated osteoblast activity *in vitro* as well as *in vivo* in rodents, leading to more bone formation. It is not yet known whether this effect is linked to the inhibition of protein prenylation in these cells, but again a new field is open for research.

* Koolwijk P, Pieterman P, Peters E, Overhand M, van der Marel GH, van Boom JH and Cohen LH, manuscript in preparation.

CONCLUDING REMARKS

Inhibitors of protein prenyl transferases interfere in cellular reactions, which are present in all types of different cells. Therefore, it is of major importance to consider the aspect of tissue specificity or specific delivery to the sites where they should act. One can think of local application, e.g. for prevention of restenosis at the site that has been dilated by balloon angioplasty, or drug targeting through modification of the inhibitors with groups that bind to specific cellular surface receptors or tissue, such as bone in the case of anti-osteoporosis and anti-bone tumour drugs. Tissue specificity was claimed for specific PFT inhibitors in mutated ras-induced tumours. However, the intracellular mechanism of action is not clear and non-ras mutated cancers seem to be sensitive as well. The vulnerability of non-transformed cells to prenylation inhibitors is not exactly predictable. Distinct signal transduction pathways may be dominant in growth stimulation of different cells, and differences in the turnover of the G-proteins involved and their sensitivity to the various inhibitors add to these uncertainties. There are indications that the growth of non-transformed cells is more dependent on geranylgeranylation than on farnesylation. It is conceivable that in certain cases a combination of compounds or compounds with less specificity, i.e. those which inhibit both PFT and geranylgeranylation, will be more effective.

Despite all these uncertainties, some of these compounds have reached clinical application in the treatment of cancer, and although their development is still in its infancy a much broader application for inhibitors of protein prenylation, for instance in the treatment of restenosis and osteoporosis [56], is anticipated. This promising future has been further extended by the notion that detrimental cell proliferation is linked to many renal disorders, leading to the hypothesis that prenylation inhibitors may also be applied in the treatment of renal diseases [57].

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