Variously Substituted (Phosphonoacetamido)Oxy Analogues of Geranylgeranyl Diphosphate (GGdP) as GGdP-transferase (GGTase) Inhibitors and Antiproliferative Agents

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Abstract: Aberrant signalling through the pathways of small GTP-binding proteins, belonging to the Ras superfamily (Ras, Rho, Rac, Cdc42 etc.), occurs in several types of cancer, where mutated Ras accumulates in its GTP-bound active form and causes uncontrolled cell proliferation. For these reasons, molecules able to target the Ras pathway in any of its stages are potentially useful in anti-cancer therapies. Inhibition of farnesyl-protein transferase (FTase), the enzyme that post-translationally activates Ras, has been pursued for the obvious role of the Ras oncoprotein in human malignancies. It was later found that some mutated forms of Ras (K- and N-Ras) can also be geranylgeranylated by geranylgeranyl-protein transferase (GGTase) when FTase is blocked, circumventing the antiproliferative effects of FTase inhibitors. Therefore, a new task has been the search for new GGTase inhibitors, which can also interfere on cell proliferation by blocking the isoprenylation of other Ras superfamily proteins (i.e. Rho, Rac, Cdc42) involved in the regulation of cell cycle progression.

We have recently described a series of phosphonoacetamido- and phosphonoacetamidoxy-stable analogues of geranylgeranyl-diphosphate (GGdP) possessing good GGTase inhibitory properties and, some of them, also remarkable GGTase/FTase selectivity levels. We have now extended this series to a larger number of variously substituted phosphonoacetamidoxy-analogues of GGdP in order to establish the effect on GGTase inhibitory activity and selectivity due to the presence of different substituents in the polar portion of these GGdP mimics. We have also measured the cytotoxicity of these compounds on tumour cell lines with the aim of evaluating their potential anti-proliferative effects.

Key Words: Geranylgeranyldiphosphate, geranylgeranyltransferase, farnesyltransferase, phosphonoacetamidoxy, enzyme inhibitors, Ras proteins, antiproliferative.

1. INTRODUCTION

Signal transduction pathways in eukaryotic cells take place by means of a post-translational prenylation of G-proteins.[1] A specific set of GTP-binding proteins, including Ras and Ras-like GTPases (Rho, Rac, Rab and Cdc42 proteins), are activated by attachment of prenyl groups (15-carbon farnesyl and 20-carbon geranylgeranyl) as a result of posttranslational modification [2]. The prenyl-transferases, which attach isoprenoid moieties to the cysteine residues near or at the carboxyl terminus of proteins, have been well characterized, and respectively termed farnesyl transferase (FTase) and geranylgeranyl transferase type I and II (GGTase I and II) [3]. The overall effect of these post-translational covalent modifications is to convert a soluble protein to a hydrophobic species that migrates and binds the cell membrane, where the process of activation proceeds. The regulatory activity of Ras-like GTPases in cellular functions, such as growth, cell motility and invasion, is critically dependent on targeting to the proper cellular membrane; indeed, protein prenylation is the first and necessary step that leads to membrane binding and acquisition of biologic activity of these proteins.

Aberrant signalling through Ras pathways occurs in several types of cancer, where mutated Ras accumulates in its GTP-bound active form and causes uncontrolled cell proliferation [4]. For these reasons, the development of specific therapeutic agents that interfere with the function of these proteins would be an appealing goal as potential treatment of tumor pathologies. A great deal of attention has been focused on the inhibition of FTase, the enzyme that post-translationally activates Ras, for the obvious role of the Ras oncoprotein in human malignancies. Recent research, however, highlights that when farnesylation of Ras-like peptides, including Rho, Rac and Cdc42, is blocked, geranylgeranylation operated by GGTases may occur and

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restore uncontrolled cell proliferation [5]. Therefore, interest towards GGTase inhibitors as potential therapeutic agents has sensibly increased and several molecules possessing GGTase inhibitory properties have been recently described [6-11].

We had already reported the development of GGTase inhibitors possessing GGdP-like structures where the metabolically unstable diphosphate unit has been replaced by other polar groups. In particular, we have recently described a series phosphonoacetamidoxy-stable analogues of GGdP (**1a-c**, Fig. **1**), that proved to be potent and selective GGTase inhibitors [12,13].

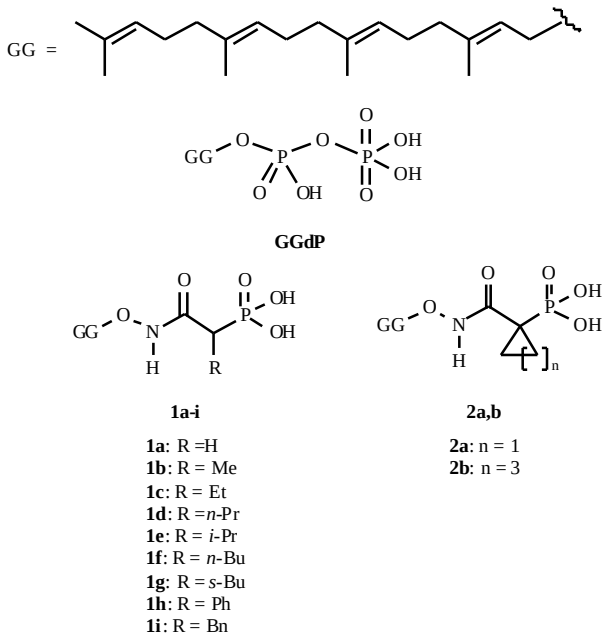
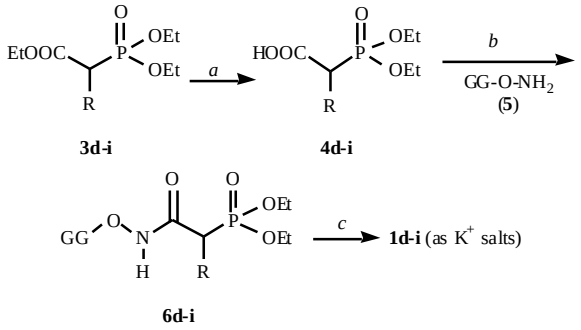


Fig. (1). Structures of GGdP, and its analogues **1a-f** and **2a,b**.

In an attempt to extend this promising series of molecules to a larger number of compounds, we wanted to explore the effect due to the introduction of a wider variety of substituents in the α-position of the phosphonoacetamidoxy moiety. We therefore synthesised and submitted to enzyme inhibition assays compounds **1d-i** and **2a,b**. Moreover, we wanted to verify the effect of both the new and the reference molecules (**1a-c**) [12,13] on tumour cell proliferation. For this reason, we also submitted the above-mentioned molecules to cytotoxicity assays on human pancreatic tumor cells Mia-PaCa-2.

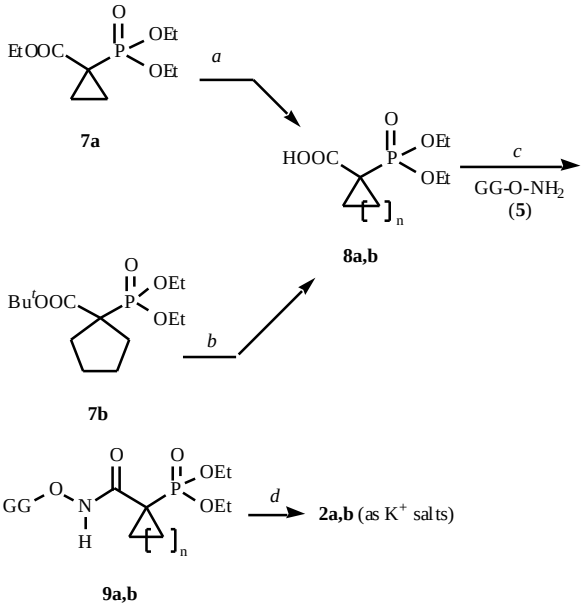
2. CHEMISTRY

Compounds **1d-i** were synthesised as shown in Scheme **1**. The appropriate ester intermediate (**3d-g** [14], **3h** [15], or **3i** [16]) was submitted to alkaline hydrolysis with a 5M aqueous potassium hydroxide solution, followed by an acidic workup, affording carboxylic acids **4d-i**. Subsequent condensation with geranylgeranyloxyamine (**5**) [12] in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole yielded phosphonates **6d-i**. Final hydrolysis of the phosphonate esters was achieved upon an initial treatment with bromotrimethylsilane and 2,4,6-collidine, followed by aqueous potassium hydroxide, to obtain final compounds **1d-i** as the dipotassium salts.



Scheme 1. Reagents and Conditions: (a) 1) 5M KOH; 2) H_3O^+. (b) 1-Hydroxybenzotriazole, EDC, THF, RT. (c) 1) TMS-Br, 2,4,6-collidine, CH_2Cl_2, RT; 2) 1N aqueous KOH.

The synthesis of cyclopropylidene (**2a**) and cyclopentylidene (**2b**) derivatives is described in Scheme **2**. Carboxylic acid derivative **8a** was obtained by alkaline hydrolysis of ethyl ester **7a** [17], after acidic workup of the reaction mixture. On the other hand, compound **8b** was obtained starting from *tert*-butyl ester **7b** [17] upon treatment with trifluoroacetic acid in the presence of anisole as the carbocation scavenger. Subsequent condensation of **8a** and **8b** with geranylgeranyloxyamine (5) [12], promoted by EDC and 1-hydroxybenzotriazole afforded oxyamides **9a** and **9b**. The ethyl phosphonate groups of **9a,b** were removed, as described above, with bromotrimethylsilane and 2,4,6-collidine. Treatment with potassium hydroxide afforded final products **2a,b** as the dipotassium salts.



Scheme 2. Reagents and Conditions: (a) 1) 5M KOH; 2) H_3O^+. (b) Trifluoroacetic acid, anisole. (c) 1-Hydroxybenzotriazole, EDC, THF, RT. (d) 1) TMS-Br, 2,4,6-collidine, CH_2Cl_2, RT; 2) 1N aqueous KOH.

3. RESULTS AND DISCUSSION

The *in vitro* inhibition assays of FTase and GGTase I were carried out by measuring the [^{3}H]GGdP and [^{3}H]FdP incorporated into H-Ras-CVLL and H-Ras-CVLS, respectively, as described in *§ 4.2.2*. The inhibitory activities of newly synthesised compounds **1d-i** and **2a,b**, together with that of **1a-c** [13], are reported in Table **1** as IC_{50}'s, the concentrations at which GGTase and FTase activities were inhibited by 50 %.

The effect of **1a-i** and **2a,b** on cell proliferation has been then evaluated by studying their cytotoxicity on the human pancreas cancer Mia-PaCa-2 cell line following a method previously described [18]. We used 5-fluorouracil (5-FU), a widely utilised chemotherapeutic agent for the treatment of gastrointestinal malignancies, as the reference compound in the cytotoxicity assay. The results of this research are shown in Table **1** where the IC_{50} values (the concentration that produces 50% reduction in cell growth) are reported.

Among the newly synthesised compounds (**1d-i** and **2a,b**), the best GGTase inhibitory properties were found with the *n*-propyl (**1d**) and benzyl (**1i**) derivatives, which displayed IC_{50} values of 623 nM and 745 nM, respectively. An accompanying significant inhibitory activity towards FTase was also found with compound **1d** (IC_{50} = 790 nM), whereas **1i** had practically no activity on this enzyme (IC_{50} > 10 μM).

As regards the cytotoxicity assays on human pancreatic tumour cells Mia-PaCa-2, the most active compound resulted to be the ethyl-substituted one (**1c**), with an IC_{50} of 1.3 μM. Comparable activities were found with all the compounds bearing no (**1a**) or small (**1b**, **1d**) α-substituents, and, surprisingly, also with α-benzylated analogue **1i**.

These results show that the introduction of alkyl substituents in the α-position are tolerated in terms of GGTase inhibitory activity only when the substituents are of limited size. In fact, the highest inhibition levels are achieved with the unsubstituted (**1a**), methyl (**1b**), ethyl (**1c**), and, to a smaller extent, *n*-propyl (**1d**) substituted derivatives. These compounds show IC_{50}'s comparable to some of the most active GGTase inhibitors so far reported [6-11]. However, a good GGTase/FTase selectivity is only found with the "smaller" compounds **1a-c**, whereas **1d** shows the same level of inhibition potency with both the enzymes. More hindered

Table 1. Inhibitory Activity of Compounds 1a-i and 2a,b on Geranylgeranyl Protein Transferase I (GGTase I) and Farnesyl Protein Transferase (FTase), Together with Cytotoxicity on Human Pancreatic Tumor Cells Mia-PaCa-2.

Compound	IC_{50} (nM)[a]		Selectivity[b]	Cytotoxicity[c] (μM)
	GGTase I	FTase		
1a	66 ± 8[d]	3500 ± 700[d]	53	2.1
1b	151 ± 30[d]	> 10000[d]	> 66	5.9
1c	146 ± 20[d]	> 10000[d]	> 66	1.3
1d	623 ± 78	790 ± 65	1.3	7.2
1e	3500 ± 400	917 ± 70	0.26	17.4
1f	> 10000	3500 ± 400	< 0.35	19.4
1g	5000 ± 650	8000 ± 900	1.6	16.5
1h	> 10000	3500 ± 450	< 0.35	> 30
1i	745 ± 38	> 10000	> 13	4.3
2a	9800 ± 1800	4500 ± 400	0.46	> 30
2b	1950 ± 250	4500 ± 370	2.3	10.3
5-FU[e]	-	-	-	9.8

[a] Values are reported as the mean ± range or SD of 2-3 independent experiments.

[b] Fold (GGTase over FTase). [c] Concentration of the compound able to reduce by 50% the tumor cell proliferation when compared to untreated control cells. [d] Reference [13]. [e] 5-Fluorouracil (5-FU) was used as the reference compound in the cytotoxicity assay.

substituents such as *i*-propyl (**1e**), *n*-butyl (**1f**) and *s*-butyl (**1g**) are detrimental for the GGTase inhibition of these compounds. The introduction of a benzyl substituent in the α-position gave a compound (**1i**) endowed of a GGTase inhibitory activity comparable to that of **1d**, and a much higher GGTase/FTase selectivity level. As a matter of fact, **1i** turned out to be the most GGTase-selective compound of the new series, showing that the presence of a phenyl ring, able to produce π-interactions with the enzyme, is well tolerated in terms of GGTase inhibitory properties. On the other hand, when the phenyl group is directly linked to the α-position of the phosphonate moiety as in compound **1h**, the activity is completely lost, thus showing that the α-position is very sensitive to any increase in steric hindrance of spatially close substituents. This trend is also confirmed by the poor inhibitory properties shown by cyclopropyl (**2a**) and cyclopentyl (**2b**) derivatives.

Interestingly, the cytotoxicity levels of the compounds tested on pancreatic tumour cells Mia-PaCa-2 displayed a certain parallelism with respect to GGTase inhibition levels. In fact, the compounds that exerted the highest inhibition degrees of cell proliferation were the ones endowed of the best GGTase inhibitory properties, such as unsubstituted **1a** (2.1 μM), methyl-substituted **1b** (5.9 μM), ethyl-substituted **1c** (1.3 μM), *n*-propyl-substituted **1d** (7.2 μM), and benzyl-substituted **1i** (4.3 μM). It should be noticed that these cytotoxic effects are superior to that of 5-FU (IC_{50} = 9.8 μM), one of the most largely used drugs in the chemotherapy of gastrointestinal tumorus.

In conclusion, we have synthesised variously substituted phosphonoacetamidoxy analogues of GGdP and we have measured their inhibition properties against GGTase and FTase. The results showed that only small alkyl substituents or a benzyl substituent are tolerated in the α-position of the polar portion of the molecule. Moreover, we have herein reported for the first time a study on the cytotoxicity exerted by the whole series of compounds on a pancreatic tumour cell line. The results seem to confirm a strict relationship between the ability of the molecules to inhibit geranylgeranylation of Ras proteins and the reduction of cell proliferation in tumours.

4. EXPERIMENTAL

4.1. Chemistry

4.1.1. General

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. ^{1}H NMR spectra of all compounds were obtained with a Varian Gemini-200 instrument operating at 200 MHz; the data are reported as follows: chemical shift (in ppm) from the Me_4Si line as the external standard, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, m = multiplet). Mass spectra were recorded on a VG 70-250S mass spectrometer or a HP-5988 A spectrometer. Analytical TLCs were carried out on 0.25-mm layer silica gel plates containing a fluorescent indicator; spots were detected under UV light (254 nm). Column chromatography was performed using 230–400 mesh silica gel. Sodium sulfate was always used as the drying agent. Evaporations were performed in vacuo (rotating evaporator). Commercially available chemicals were purchased from Sigma-Aldrich.

4.1.2. Synthesis of 4d-i and 8a

The appropriate α-substituted triethyl phosphonoacetate ester (**3d-g** [C3], **3h** [C4], **3i** [C5], or **7a** [C6]) (5.0 mmol) was treated for 18 hours at RT with an equimolar amount of a 85% solution of KOH in H_2O/EtOH (5:2 ratio). The reaction mixture was then concentrated to dryness and the residue was taken up in water. The resulting solution was acidified with a 10% aqueous solution of HCl to pH 1, then it was saturated with solid NaCl, and finally it was extracted several times with dichloromethane. The combined organic phase were dried and concentrated to obtain the carboxylic acid derivatives **4d-j**.

4d: 70% yield; ^{1}H-NMR ($CDCl_3$) δ 0.92 (t, 3H, *J* = 7.2 Hz), 1.33 (t, 6H, *J* = 7.2 Hz), 1.55-2.15 (m, 4H), 2.98 (ddd, 1H, *J* = 22.0, 10.2, 5.1 Hz), 3.97-4.38 (m, 4H). MS *m/e* 239 $(M+H)^+$.

4e: 73% yield; ^{1}H-NMR ($CDCl_3$) δ 0.90-1.50 (m, 12H), 2.10-2.90 (m, 2H), 3.88-4.40 (m, 4H). MS *m/e* 239 $(M+H)^+$.

4f: 86% yield; ^{1}H-NMR ($CDCl_3$) δ 0.89 (t, 3H, *J* = 6.8 Hz), 1.33 (t, 6H, J = 7.2Hz), 1.10-2.00 (m, 6H), 2.92 (ddd, 1H, *J* = 22.1, 10.2, 5.3 Hz), 3.90-4.40 (m, 4H). MS *m/e* 253 $(M+H)^+$.

4g: 75% yield; ^{1}H-NMR ($CDCl_3$) δ 0.90 (t, 3H, *J* = 6.8 Hz), 1.00-1.20 (m, 6H), 1.33 (t, 6H, *J* = 7.2 Hz), 2.85 (ddd, 1H, *J* = 21.8, 8.6, 5.0 Hz), 4.19 (q, 4H, *J* = 7.2 Hz). MS *m/e* 253 $(M+H)^+$.

4h: 73% yield; ^{1}H-NMR ($CDCl_3$) δ 1.16 (t, 3H, *J* = 7.2 Hz), 1.25 (t, 3H, *J* = 7.2 Hz), 3.75-4.30 (m, 5H), 7.10-7.60 (m, 5H). MS *m/e* 273 $(M+H)^+$.

4i: 81% yield; ^{1}H-NMR ($CDCl_3$) δ 1.27 (t, 3H, *J* = 7.2 Hz), 1.30 (t, 3H, *J* = 7.2 Hz), 2.95-3.50 (m, 3H), 3.75-4.35 (m, 4H), 7.00-7.30 (m, 5H). MS *m/e* 287 $(M+H)^+$.

8a: 57% yield; ^{1}H-NMR ($CDCl_3$) δ 1.33 (t, 6H, *J* = 7.2 Hz), 1.10-1.75 (m, 4H), 3.92-4.45 (m, 4H). MS *m/e* 205 $(M-OH)^+$.

4.1.3. Synthesis of 8b

A solution of ester 7b [C6] (0.52g, 1.70 mmol) in anhydrous dichloromethane (20 mL) was added dropwise to a cooled (0 °C) solution of trifluoroacetic acid in anhydrous dichloromethane (20 mL). The resulting mixture was stirred for 48 hours at RT. After this period, the solvent was removed under vacuum. The residue was dissolved in ethyl acetate (25 mL) and the resulting solution was washed with an ice-cold saturated solution of sodium bicarbonate. The alkaline water phase was acidified to pH 3 with 10% HCl and then extracted with ethyl acetate. The organic phase was then dried and concentrated under vacuum, to afford compound **8b** (0.41 g, 1.6 mmol, 86% yield): ^{1}H-NMR ($CDCl_3$) δ 1.32 (t, 6H, *J* = 7.2 Hz), 1.52-1.88 (m, 4H), 1.92-2.46 (m, 4H), 3.98-4.39 (m, 4H). MS *m/e* 233 $(M-OH)^+$.

4.1.4. Synthesis of 6d-i and 9a,b

A solution of geranylgeranyloxyamine **5** (0.40 g, 1.3 mmol) in anhydrous THF (20 mL) was treated with the appropriate α-substituted phosphonoacetic acid (**4d-i** or **8a,b**) (1.4 mmol) in the presence of 1-hydroxybenzotriazole (0.27 g, 2.0 mmol), and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (0.30 g, 1.6 mmol). The resulting reaction mixture was stirred at RT for 18 hours. The solvent was then removed under vacuum and the crude products were purified by silica gel column chromatography eluting with hexane/EtOAc mixtures, to obtain phosphonoacetamidoxy esters **6d-i** and **9a,b**.

6d: 66% yield; ^{1}H-NMR ($CDCl_3$) δ 0.90 (t, 3H, *J* = 6.4 Hz), 1.31 (t, 6H, *J* = 7.2 Hz), 1.50-1.73 (m, 15H), 1.90-2.20 (m, 16H), 2.50-2.72 (m, 1H), 3.90-4.32 (m, 4H), 4.40 (d, 2H, *J* = 7.2 Hz), 5.10 (br, 3H), 5.39 (t, 1H, *J* = 7.2 Hz). MS (FAB^+) *m/e* 526 $(M+H)^+$.

6e: 56% yield; ^{1}H-NMR ($CDCl_3$) δ 0.88-1.40 (m, 12H), 1.48-1.78 (m, 15H), 1.90-2.20 (m, 14H), 3.85-4.32 (m, 4H), 4.41(d, 2H, *J* = 7.2 Hz), 5.06 (br, 3H), 5.39 (t, 1H, *J* = 7.2 Hz). MS (FAB^+) *m/e* 526 $(M+H)^+$**.**

6f: 84% yield; ^{1}H-NMR ($CDCl_3$) δ 0.91 (t, 3H, *J* = 6.4 Hz), 1.10-1.45 (m, 6H), 1.50-1.80 (m, 15H), 1.90-2.20 (m, 18H), 2.30-2.50 (m, 1H), 3.87-4.32 (m, 4H), 4.41 (d, 2H, *J* = 7.2 Hz), 5.11 (br, 3H), 5.38 (t, 1H, *J* = 7.2 Hz). MS (FAB^+) *m/e* 540 $(M+H)^+$**.**

6g: 60% yield; ^{1}H-NMR ($CDCl_3$) δ 0.70-1.18 (m, 6H), 1.33 (t, 6H, *J* = 7.2 Hz), 1.50-1,80 (m, 15H), 1.90-2.20 (m, 15H), 2.50-2.90 (m, 1H), 3.90-4.32 (m, 4H), 4.42 (d, 2H, *J* = 7.2 Hz), 5.09 (br, 3H), 5.38 (t, 1H, *J* = 7.2 Hz). MS (FAB^+) *m/e* 540 $(M+H)^+$.

6h: 61% yield; ^{1}H-NMR ($CDCl_3$) δ 1.21-1.40 (m, 6H), 1.49-1.73 (m, 15H), 1.76-2.20 (m, 12H), 3.12 (d, 1H, *J* = 19.0 Hz), 3.66-4.20 (m, 4H), 4.37 (d, 2H, *J* = 7.2 Hz), 5.10 (br, 3H), 5.33 (t, 1H, *J* = 7.2 Hz), 7.11-7.51 (m, 5H). MS (FAB^+) m/e 560 $(M+H)^+$.

6i: 53% yield; ^{1}H-NMR ($CDCl_3$) δ 1.10-1.35 (m, 6H), 1.47-1.78 (m, 15H), 1.90-2.20 (m, 12H), 2.60 (m, 1H), 3.90-4.50 (m, 8H), 5.09 (br, 3H), 5.31 (t, 1H, *J* = 7.2 Hz), 7.10-7.30 (m, 5H). MS (FAB^+) *m/e* 574 $(M+H)^+$**.**

9a: 65% yield; ^{1}H-NMR ($CDCl_3$) δ 1.32 (t, 6H, *J* = 7.2 Hz), 1.47-1.80 (m, 15H), 1.92-2.30 (m, 16H), 4.05 (q, 2H, *J* = 7.2 Hz), 4.15 (q, 2H, *J* = 7.2 Hz), 4.40 (d, 2H, *J* = 7.2 Hz), 5.05 (br, 3H), 5.30 (t, 1H, *J* = 7.2 Hz). MS (FAB^+) *m/e* 510 $(M+H)^+$**.**

9b: 51% yield; ^{1}H-NMR ($CDCl_3$) δ 1.31 (t, 6H, *J* = 7.2 Hz), 1.49-1.82 (m, 15H), 1.89-2.34 (m, 20H), 3.86-4.23 (m, 6H), 4.92-5.16 (m, 3H), 5.30 (t, 1H, *J* = 7.2 Hz). MS (FAB^+) *m/e* 538 $(M+H)^+$.

4.1.5. Synthesis of the Dipotassium Salt of Derivatives 1d-i and 2a,b

Bromotrimethylsilane (0.46 mL, 3.5 mmol) was added to a stirred solution of the appropriate diethyl phosphono-acetamidoxy ester (**6d-i** or **9a,b**) (0.70 mmol) in anhydrous dichloromethane (8 mL). The resulting mixture was stirred at RT for 18 hours. After evaporation of the solvent, the residue was treated with a 1N aqueous solution of KOH (5 mL) and the resulting mixture was stirred at RT for 3 hours. After removal of water under high vacuum, the crude residue was purified by column chromatography on reverse phase silica gel (Merck Lichroprep® RP-18) eluting with MeOH-H_2O. The appropriate fractions were combined, evaporated, lyophilized, and pump-dried to give final products **1d-i** and **2a,b** as the dipotassium salts.

1d: 56% yield; ^{1}H-NMR (CD_3OD) δ 0.90 (t, 3H, *J* = 6.4 Hz), 1.15-1.32 (m, 4H), 1.49-1.78 (m, 15H), 1.80-2.20 (m, 12H), 2.52 (m, 1H), 4.35 (d, 2H, *J* = 7.2 Hz), 5.08 (br, 3H), 5.38 (t, 1H, *J* = 7.2 Hz). MS (FAB^+) *m/e* 546 $(M+H)^+$.

1e: 59% yield; ^{1}H-NMR (CD_3OD) δ 0.85-1.20 (m, 7H), 1.45-1.75 (m, 15H), 1.90-2.20 (m, 12H), 2.52 (m, 1H), 4.35 (d, 2H, *J* = 7.2 Hz), 5.07 (br, 3H), 5.30 (t, 1H, *J* = 7.2 Hz). MS (FAB^+) *m/e* 546 $(M+H)^+$.

1f: 36% yield; ^{1}H-NMR (CD_3OD) δ 0.91 (t, 3H, *J* = 6.4 Hz), 1.12-1.45 (m, 6H), 1.51-1.78 (m, 15H), 1.90-2.20 (m, 12H), 2.53 (m, 1H), 4.38 (d, 2H, *J* = 7.2 Hz), 5.07 (br, 3H), 5.40 (t, 1H, *J* = 7.2 Hz). MS (FAB^+) *m/e* 560 $(M+H)^+$.

1g: 42% yield; ^{1}H-NMR (CD_3OD) δ 0.70-1.37 (m, 9H), 1.51-1.75 (m, 15H), 1.90-2.20 (m, 12H), 2.55 (m, 1H), 4.37 (d, 2H, *J* = 7.2 Hz), 5.09 (br, 3H), 5.35 (t, 1H, J = 7.2 Hz). MS (FAB^+) *m/e* 560 $(M+H)^+$.

1h: 60% yield; ^{1}H-NMR (CD_3OD) δ 1.47-1.78 (m, 15H), 1.92-2.20 (m, 12H), 3.72 (d, 1H, *J* = 19.2 Hz), 4.39 (d, 2H, *J* = 7.2 Hz), 5.10 (br, 3H), 5.31 (t, 1H, *J* = 7.2 Hz), 7.10-7.65 (m, 5H). MS (FAB^+) *m/e* 580 $(M+H)^+$.

1i: 37% yield; ^{1}H-NMR(CD_3OD) δ 1.49-1.80 (m, 15H), 1.90-2.15 (m, 12H), 2.60 (m, 1H), 4.00-4.30 (m, 4H), 5.08 (br, 3H), 5.32 (t, 1H, J=7.2 Hz), 7.02-7.29 (m, 5H). MS (FAB^+) *m/e* 594 $(M+H)^+$.

2a: 54% yield; ^{1}H-NMR (CD_3OD) δ 1.53-1.76 (m, 15H), 1.92-2.15 (m, 16H), 4.35 (d, 2H, *J* = 7.2 Hz), 5.05 (br, 3H), 5.30 (t, 1H, *J* = 7.2 Hz). MS (FAB^+) *m/e* 530 $(M+H)^+$.

2b: 36% yield; ^{1}H-NMR (CD_3OD) δ 1.51-1.83 (m, 15H), 1.92-2.36 (m, 20H), 4.37 (d, 2H, *J* = 7.2 Hz), 4.92-5.16 (m, 3H), 5.30 (t, 1H, *J* = 7.2 Hz). MS (FAB^+) m/e 558 $(M+H)^+$.

4.2. Biological Assays

4.2.1. Materials

[^{3}H]GGPP (specific activity 22 Ci/mmol) and [^{3}H]FdP (specific activity 16 Ci/mmol) were purchased from Perkin-Elmer Life Science; GGTase I and FTase were purchased from Sigma-Aldrich Corporation; H-Ras-CVLL, H-Ras-CVLS (Wild type), and Zwittergent 3-12 were purchased from Calbiochem. All other reagents were obtained from normal commercial sources.

The human pancreas cancer cell line MIA PaCa-2 (American Type Culture Collection, USA) derived from exocrine pancreas neoplasm was cultivated in Dulbecco's modified Eagle's medium (87.5%), fetal bovine serum (10%) and horse serum (2.5%). Cells were routinely subcultured when 75% confluent at a ratio of 1:15 by treatment with 1.5

mM EDTA and plated in a 75 cm^2 culture flasks. Cells were incubated in 5% CO_2/95% air at 37 °C.

4.2.2. GGTase I and FTase Activity Assay

In vitro inhibition studies were performed as previously described [19,20], with some modifications. Briefly, GGTase (250 ng) and FTase (20 ng) were incubated in 50 mM Tris-HCl, pH 7.7, 25 μM $ZnCl_2$, 20 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 0.5 mM Zwittergent 3-12, in the presence of different concentrations of inhibitors in a final volume of 50 μl. The reactions were incubated at 30°C for 30 min with recombinant H-Ras-CVLL (2.5 μM) and [^{3}H]GGdP (0.1 μM) for GGTase I, and recombinant H-Ras-CVLS (2.5 μM) and [^{3}H]FdP (0.6 μM) for FTase. After incubation the reaction was stopped and filtered on glass fiber filters to separate free from incorporated label. The activity of the inhibitors is reported in Table **1** as IC_{50}, the concentration at which GGTase and FTase activity was inhibited by 50 %.

4.2.3. Cytotoxicity Assay

The growth inhibitory effect of GGPP analogues was assayed by direct cell count [18]. Briefly, a single cell suspension was prepared by trypsinisation and cells counted by haemocytometry; approximately 2×10^3 cells were resuspended in 0.9 ml of the specific culture medium for the cell line. The cells were seeded into each well of a 24-multiwell plate, and incubated at 37°C with 5% CO_2. Twenty-four hours later 100 μl of complete medium containing GGPP analogues at appropriate concentrations (0.001-50 μM) were added. Cells were also treated with 5-fluorouracil (5-FU) as a reference drug at the same concentrations. After a 72 h-exposure to the test compounds, culture medium was removed, cell harvested by trypsinisation and counted by haemocytometry. Cell growth inhibition was expressed as the percentage of control (no drugs) cell number and the 50% inhibitory concentration of cell growth (IC_{50}) was calculated by non-linear least squares curve fitting.

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