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Bioactivity of glycogen phosphorylase inhibitors that bind to the purine nucleoside site

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Abstract—The bioactivity in hepatocytes of glycogen phosphorylase inhibitors that bind to the active site, the allosteric activator site and the indole carboxamide site has been described. However, the pharmacological potential of the purine nucleoside inhibitor site has remained unexplored. We report the chemical synthesis and bioactivity in hepatocytes of four new olefin derivatives of flavopiridol (**1**–**4**) that bind to the purine site. Flavopiridol and **1**–**4** counteracted the activation of phosphorylase in hepatocytes caused by AICAR (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside), which is metabolised to an AMP analogue. Unlike an indole carboxamide inhibitor, the analogues **1** and **4** suppressed the basal rate of glycogenolysis in hepatocytes by allosteric inhibition rather than by inactivation of phosphorylase, and accordingly caused negligible stimulation of glycogen synthesis. However, they counteracted the stimulation of glycogenolysis by dibutyryl cAMP by both allosteric inhibition and inactivation of phosphorylase. Cumulatively, the results show key differences between purine site and indole carboxamide site inhibitors in terms of (i) relative roles of dephosphorylation of phosphorylase-a as compared with allosteric inhibition, (ii) counteraction of the efficacy of the inhibitors on glycogenolysis by dibutyryl-cAMP and (iii) stimulation of glycogen synthesis.

1. Introduction

Increased hepatic glucose production by glycogenolysis and gluconeogenesis is a contributing factor to the hyperglycaemia in Type-2 diabetes. Inhibition of hepatic glycogen phosphorylase, the flux-generating enzyme in glycogenolysis, is therefore considered a potential therapeutic strategy for controlling blood glucose in Type-2 diabetes.[1]

Hepatic glycogen phosphorylase is regulated by covalent modification (phosphorylation of a serine residue at the N-terminus) and by allosteric mechanisms.[2] The dephosphorylated form of liver glycogen phosphorylase (phosphorylase-b) is essentially inactive in liver in vivo and only the phosphorylated form (phosphorylase-a) contributes to glycogenolysis.[3] Phosphorylation is catalysed by phosphorylase kinase which is activated by cAMP and elevated calcium ion concentration and dephosphorylation is catalysed by the catalytic unit of protein phosphatase-1 in association with glycogen targeting proteins.[4] Phosphorylase exists as either a relaxed (R) or a tense (T) conformation representing active and inactive conformations. Phosphorylation and certain ligands (AMP, phosphate and glucose 1-P) favour the

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside; DAB, 1,4-dideoxy-1,4-imino-D-arabinitol; DMEM, Dulbecco's modified Eagle's medium; glucose 1-P, glucose 1-phosphate; glucose 6-P, glucose 6-phosphate; MEM, Minimum essential medium; ZMP, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranotide.

Keywords: Type-2 diabetes; Glycogen phosphorylase; Flavopiridols; Inhibitors; Glycogen metabolism; Hepatocytes.

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R-conformation, whereas glucose and glucose 6-P favour the T-conformation, which is a better substrate for dephosphorylation by protein phosphatase-1.[5–7]

In the liver the phosphorylated form of phosphorylase is a potent inhibitor of glycogen synthase phosphatase through interaction with a high-affinity allosteric site on the glycogen targeting protein G_L, which binds protein phosphatase-1.[2] Depletion of phosphorylase-a by dephosphorylation induced by glucose, glucose 6-P or pharmacological ligands that favour the T-conformation, results in activation of glycogen synthase through reversal of the inhibition of glycogen synthase phosphatase.[8–10]

Various ligand binding sites have been identified from crystallography of either rabbit muscle or human liver phosphorylase that bind physiological or pharmacological ligands, they include the catalytic site, an allosteric AMP-activator site, a purine nucleoside inhibitor site (caffeine-binding site), an indole carboxamide site and a glycogen storage site.[5,11,13] The biological actions in hepatocytes of high-affinity ligands of the catalytic site, the activator site and the indole carboxamide site have been reported.[8–10,14–17] Although the caffeine-binding site is well characterised from crystallographic studies, less is known about its physiological role or pharmacological potential. Uric acid, xanthine, hypoxathine and allopurinol bind at the purine nucleoside site, but with weaker affinity than caffeine, whereas riboflavin binds with higher affinity.[18] Whether these or other endogenous ligands have a physiological role in regulating hepatic glycogenolysis is not known.[19] Flavopiridol, a potent inhibitor of cyclin-dependent kinases with antiproliferative effects on tumour cells, was recently identified as an inhibitor of glycogen phosphorylase and high-affinity ligand of the purine nucleoside site.[20,21] Flavopiridol is therefore a potential tool to determine the pharmacological actions of the purine-inhibitor site of glycogen phosphorylase in comparison with other inhibitor sites. In this study, we compared the mode of action of flavopiridol and four olefin derivatives of flavopiridol with the effects of an indole carboxamide that has been well characterised previously from in vivo and in vitro studies.[9,10,22] The results show distinct differences in the mode of action of purine nucleoside and indole carboxamide site inhibitors.

2. Results

2.1. Chemistry

The olefin analogues of flavopiridol (**1**–**4**, Fig. 1) were synthesized according to Murthi et al.[23] with minor modifications (Scheme 1). Compound **5** was prepared as described by Naik et al.[24] In general, 1-methyl-4-(2,4,6 trimethoxyphenyl)-1,2,3,6-tetrahydropyridin, **5**, was treated with BF_3OEt_2 and Ac_2O to afford 1-methyl-4-(2-hydroxy-3-acetyl-4,6-dimethoxyphenyl)-1,2,3,6-tetrahydropyridine, **6**, with selective demethylation, acylation and Lewis acid-promoted Fries rearrangement. Benzoylation of **6** with the appropriate acid

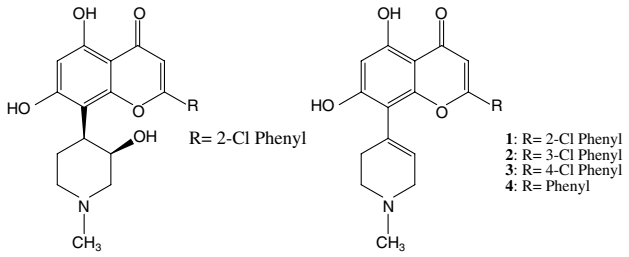


Figure 1. Flavopiridol (left) and its olefin analogues.

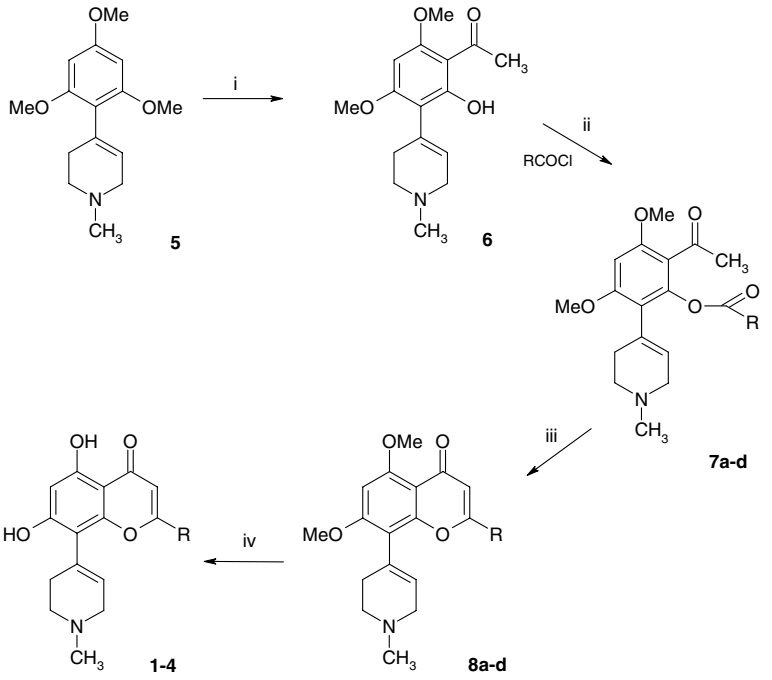
chloride catalysed by DMAP gave the ester **7a**–**d**, (Fig. 2). Reaction of the derivative **7** with NaH/THF and H_2SO_4/AcOH provided the dimethoxy flavone **8a**–**d**, (Fig. 2). These were completely demethylated to the desired final products, **1**–**4**, upon treatment with pyridinium hydrochloride. The alternative synthetic route starting with **9**,[25] has been followed as well (Scheme 2). However, acid-catalysed condensation between 3,5-dimethoxy phenol and 1-methyl-4-piperidone afforded, in addition to the desired compound, **9**, the product of double substitution at the phenyl ring, **10**, in a 19% yield (Scheme 3).

2.2. Structure and inhibitor potency of flavopiridol derivatives on muscle glycogen phosphorylase

Previous work showed that flavopiridol (Fig. 1) binds to muscle phosphorylase at the purine nucleoside inhibitor site[21] and is a potent inhibitor of the AMP-activated muscle phosphorylase-b but is less inhibitory to phosphorylase-a.[20] We tested the potency of four olefin derivatives of flavopiridol (**1**–**4**) at inhibiting muscle phosphorylase-b. The K_i values on muscle glycogen phosphorylase-b were between 0.83 and 1.89 μM (Table 1). To elucidate the mechanism of inhibition, we have determined the crystal structures of the four analogues **1**–**4** complexed with rabbit muscle glycogen phosphorylase b. The structures showed that the inhibitors bind at the allosteric inhibitor site, where flavopiridol binds (Ganotidis et al., unpublished results).

2.3. Effects of flavopiridols on glycogenolysis in hepatocytes

The efficacy of flavopiridol and the olefin analogues **1**–**4** at inhibiting glycogenolysis in hepatocytes was tested at concentrations of 2.5–25 μM and compared with two previously characterised phosphorylase inhibitors, DAB[16] and the indole carboxamide, CP 91149.[9] Glycogenolysis was determined in a glucose-free medium without or with 100 μM dibutyryl cAMP, which caused a 2.5-fold stimulation of glucose production (from 47 ± 9 to 115 ± 19 nmol mg^{-1} protein). DAB caused a similar fractional inhibition of glycogenolysis irrespective of the absence or presence of dibutyryl-cAMP (Fig. 3A), whereas CP-91149 was more potent in the absence of dibutyryl-cAMP (Fig. 3B). Compounds flavopiridol, **2** and **3** (at 25 μM) inhibited glycogenolysis by less than 35% (results not shown). In contrast **1** and **4** caused a concentration-dependent inhibition of glycogen degradation with maximum inhibition of 49% and 54% in the absence



Scheme 1. Reagents: (i) $BF_3{\cdot}OEt_2$, Ac_2O, dry CH_2Cl_2; (ii) 10% DMAP, dry pyridine; (iii) a—NaH, dry THF; b—H_2SO_4, AcOH; (iv) pyridine hydrochloride.

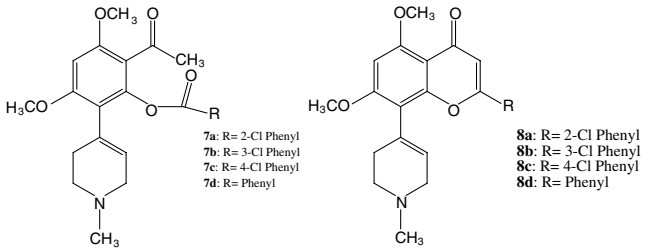


Figure 2. Intermediate products **7a–d** and **8a–d**.

of dibutyryl cAMP, and 61% and 69% in the presence of dibutyryl cAMP (Figs. 3C and D).

2.4. Effects of flavopiridols on inactivation of phosphorylase-a

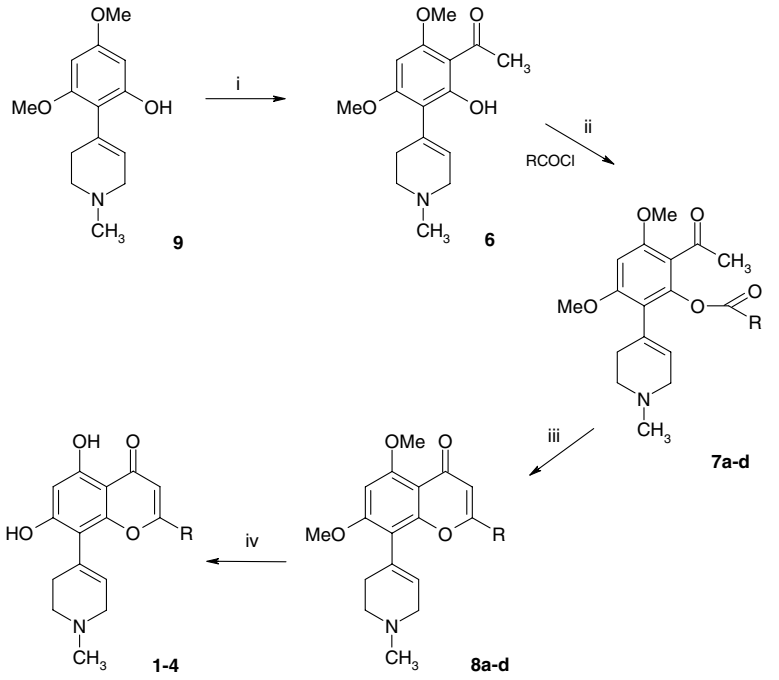
Since CP-91149 and DAB inhibit glycogenolysis by different mechanisms (dephosphorylation of phosphorylase-a and allosteric inhibition, respectively),[17] we tested the effects of flavopiridols (2.5–25 μM) on the activation state of phosphorylase (Fig. 4). Unlike CP-91149, none of the flavopiridols caused significant depletion (dephosphorylation) of phosphorylase-a in the absence of dibutyryl-cAMP stimulation at concentrations up to 25 μM (Fig. 4A). However, flavopiridol, **1**, **2** and **4** (25 μM) significantly counteracted (by 14%, 27%, 18% and 41%, respectively) the activation caused by dibutyryl cAMP (Fig. 4A). This inactivation was less than that caused by CP-91149 (70%). To test whether the flavopiridols counteract the activation of phosphorylase by an AMP analogue, we used AICAR which is metabolised by hepatocytes to the AMP analogue, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranotide (ZMP).[7,26,27] All flavopiridols tested including **3** significantly counteracted the activation of phosphorylase by 200 μM AICAR at inhibitor concentrations of 25 μM (Fig. 4B) and also at 2.5 and 10 μM ($P < 0.05$, in all cases, results not shown). The effects of varying inhibitor concentrations are shown for CP-91149 (Fig. 4C) and **4** (Fig. 4D).

2.5. Analogue 4 inhibits glycogenolysis by both allosteric inhibition and inactivation of phosphorylase-a

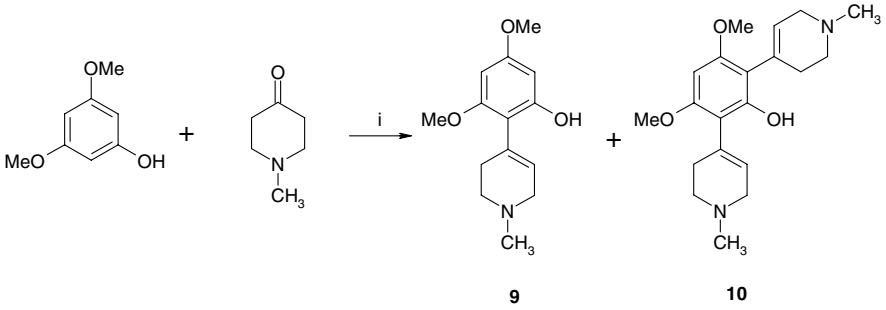
To determine the relative contributions of inactivation of phosphorylase as distinct from allosteric inhibition, we correlated the effects of CP-91149 and des-chloro **4** on glycogenolysis with the activation state of phosphorylase being determined from the activity of phosphorylase-a (data from Figs. 3 and 4, respectively). The rate of glycogenolysis decreased with increasing CP-91149 concentration (2.5–25 μM) and correlated with depletion of phosphorylase-a (Fig. 5A) as shown previously.[17] However, in the case of analogue **4** the rates of glycogenolysis were lower than could be explained by inactivation of phosphorylase when compared with the data for CP-91149 (Fig. 5B). In the absence of AICAR or dibutyryl cAMP, inhibition of glycogenolysis was predominantly due to allosteric inhibition as shown by the lack of depletion of phosphorylase-a, whereas in the presence of AICAR or dibutyryl cAMP inhibition of glycogenolysis was in part due to inactivation of phosphorylase and in part to allosteric inhibition as shown by the distribution of the data points below the correlation line corresponding to CP-91149 (Fig. 5B).

2.6. Lack of stimulation of glycogen synthesis by flavopiridols

The indole carboxamide, CP-91149, caused a 6.5-fold increase in glycogen synthesis in incubations with



Scheme 2. Reagents: (i) $BF_3{\cdot}OEt_2$, Ac_2O, dry CH_2Cl_2; (ii) 10% DMAP, dry pyridine; (iii) a—NaH, dry THF; b—H_2SO_4, AcOH; (iv) pyridine hydrochloride.



Scheme 3. Reagents: (i) HCl(g), AcOH.

Table 1. Inhibition studies of flavopiridol and compounds **1**–**4** on rabbit muscle phosphorylase-b

Compound	K_i (μM)
Flavopiridol	1.16 (±0.01)
1	0.99 (±0.16)
2	0.83 (±0.06)
3	1.89 (±0.06)
4	1.28 (±0.01)

15 mM glucose (130 ± 31 vs 19.9 ± 7.1 nmol/3 h mg^{-1}), and a 2-fold activation of glycogen synthase (results not shown) in agreement with previous findings.[10] The ortho analogue **1** caused a small (34%, $P < 0.02$) stimulation of glycogen synthesis (26.8 ± 6.7 vs 19.9 ± 7.1 nmol/3 h mg^{-1}), whereas the other flavopiridols had no effect on glycogen synthesis (results not shown). We confirmed that the flavopiridols did not affect glucose phosphorylation as determined from the metabolism of [2-^{3}H] glucose (results not shown). This indicates that the lack of stimulation of glycogen synthesis by flavopiridols cannot be explained by either inhibition of glucokinase or by non-specific cytotoxicity.

3. Discussion

The activity of phosphorylase-a and also its dephosphorylation by protein phosphatase-1 are regulated by multiple interacting allosteric sites. The main physiological regulators of hepatic phosphorylase are glucose and glucose 6-P which act synergistically in promoting inactivation (dephosphorylation).[6,7,28] Glucose inhibits purified phosphorylase synergistically with pharmacological ligands of the purine nucleoside site, the indole carboxamide site and the AMP-site.[5,9,29,30] Glucose 6-P promotes inactivation of phosphorylase in hepatocytes synergistically with an inhibitor of the indole carboxamide site.[31] Although intermediates of the purine catabolism are low-affinity inhibitors of the purine nucleoside site,[18,19] whether they have a physiological role in the control of either activity or activation state of phosphorylase is not

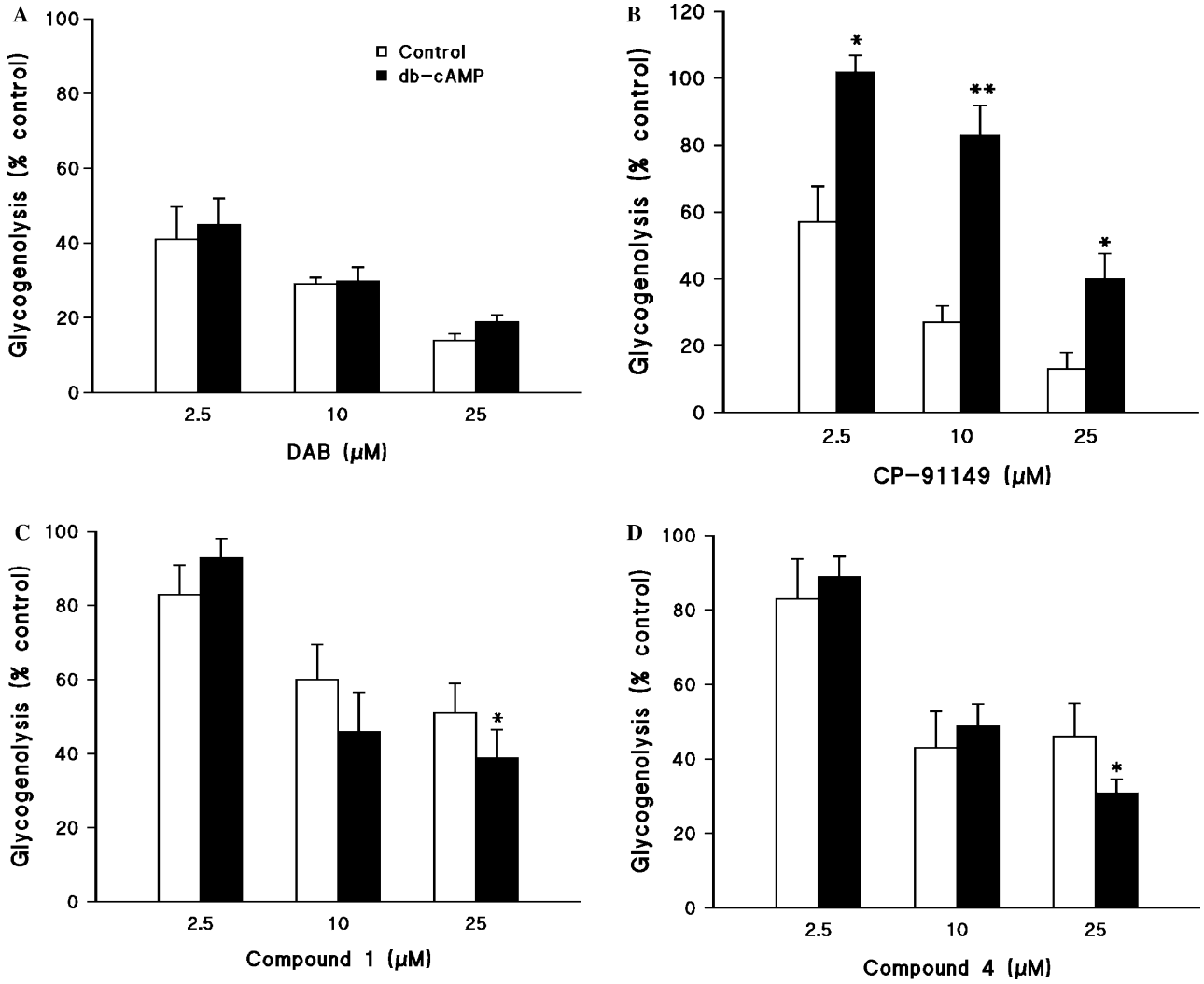


Figure 3. Inhibition of glycogenolysis. Hepatocytes were pre-cultured in MEM containing 25 mM glucose and 100 nM insulin for 16 h and were then incubated in glucose-free DMEM in either the absence (open bars) or presence (solid bars) of 100 μM dibutyryl cAMP (db-cAMP) and the inhibitors indicated: A, DAB; B, CD-91149; C, Compound 1; D, Compound 4. Rates of glucose production in incubations with inhibitors are expressed as percentage of control without inhibitor. Results are means ± SE for five experiments. $^{*}P < 0.05$; $^{**}P < 0.005$ presence versus absence of dibutyryl cAMP.

known. Flavopiridol, a cyclin-dependent kinase inhibitor, is the highest-affinity pharmacological ligand of the purine nucleoside site reported.[20,21] Derivatives of flavopiridol are therefore potential tools to compare the mechanism of action of the purine nucleoside site with the high-affinity ligands of other allosteric sites such as the catalytic site[16] and the indole carboxamide site.[9,11,12] In this study, we report the synthesis and bioactivity of four new olefin derivatives of flavopiridol.

Previous studies comparing the mechanism of action of the indole carboxamide inhibitor, CP-91149,[9] with DAB[16] showed that the latter compound inhibited glycogenolysis exclusively by allosteric inhibition, whereas the indole carboxamide inhibited predominantly by dephosphorylation of phosphorylase.[17] Furthermore, the indole carboxamide, but not DAB, showed a lower potency at inhibiting glycogenolysis in the presence of glucagon. This was explained by opposing effects of phosphorylase kinase (activated by cAMP) which catalyses conversion of phosphorylase b to phosphorylase-a and the indole carboxamide which makes phosphorylase-a a better substrate for dephosphorylation.

We show in this study that four derivatives of flavopiridol, with similar K_i values of 1–2 μM, affect the activation state of glycogen phosphorylase and glycogenolysis by different mechanisms from the indole carboxamide or DAB. Unlike the indole carboxamide they had negligible effect on the activation state of glycogen phosphorylase in the absence of AICAR or dibutyryl cAMP, and accordingly they had negligible effect on glycogen synthesis, since stimulation by indole carboxamides is explained by depletion of phosphorylase-a which causes sequential activation of glycogen synthase.[10] However, all five flavopiridols fully blocked the activation of phosphorylase caused by AICAR and they partially reversed the activation caused by dibutyryl cAMP. The latter effects suggest suppression of phosphorylase kinase activity through either a substrate-independent mechanism as suggested by Kaiser et al.[20] or alternatively through a

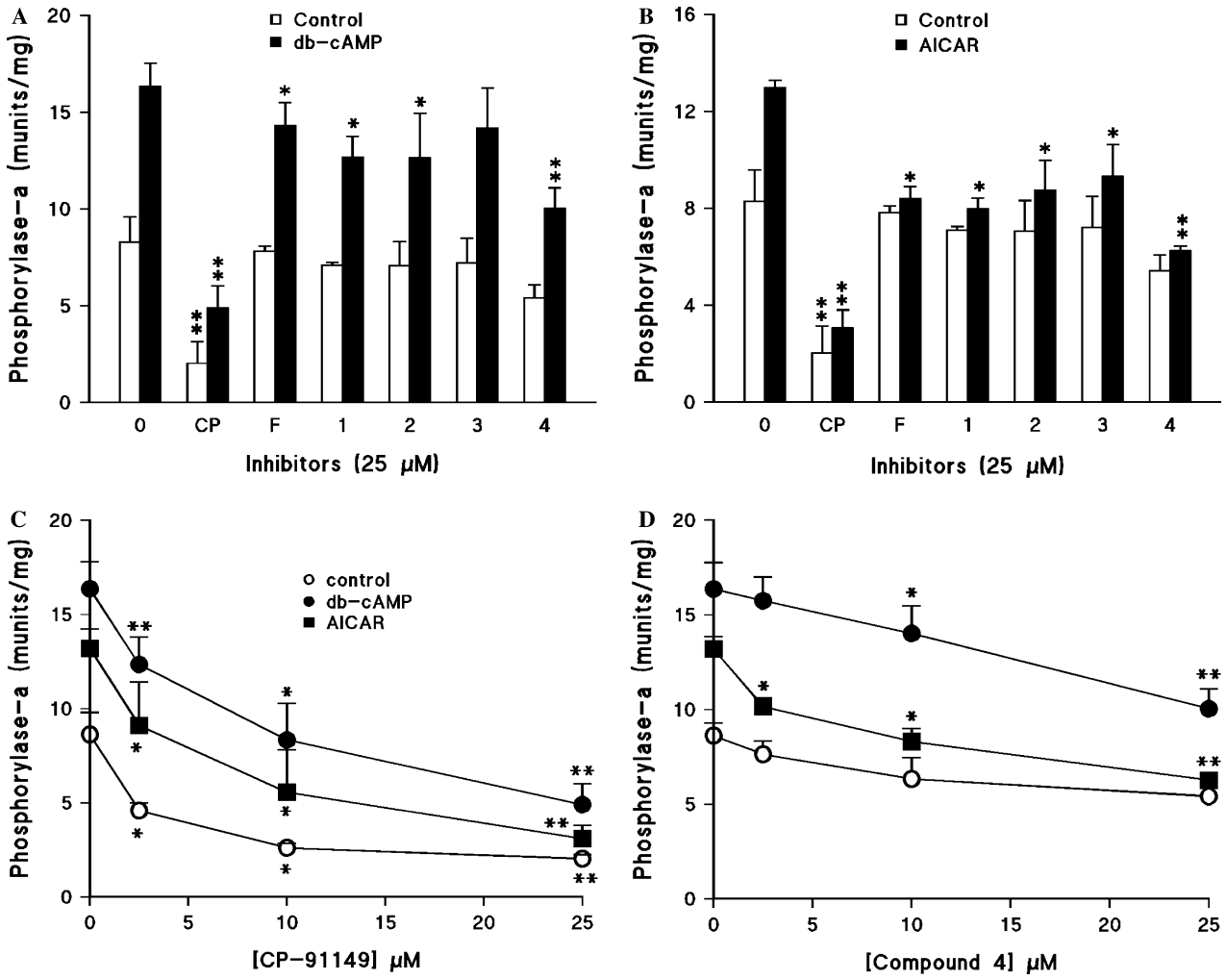


Figure 4. Effects of inhibitors on conversion of phosphorylase-a to phosphorylase-b in hepatocytes. Hepatocytes pre-cultured as in Figure 3 were pre-incubated with inhibitors (flavopiridol, **F** or compounds **1**–**4**) for 30 min in glucose-free DMEM, then dibutyryl cAMP or AICAR was added as indicated and incubations continued for a further 30 min before snap-freezing in liquid nitrogen for determination of phosphorylase-a. (A and B) Hepatocytes were incubated without (0) or with 25 μM inhibitor followed by the absence (open bars) or presence (solid bars) of 100 μM dibutyryl-cAMP (A) or 200 μM AICAR (B). (C and D) Effects of varying concentration of CP-91149 (C) or analogue **4** (B) followed by the absence (O) or presence of 100 μM dibutyryl-cAMP (filled circle) or 200 μM AICAR (filled square). Results are means ± SE for four experiments. $*P < 0.05$; $**P < 0.005$ relative to no inhibitor.

substrate-directed mechanism by favouring the T-state as suggested for glucose 6-P.[32,33] AICAR is metabolised by hepatocytes to ZMP, an AMP analogue,[26] and promotes the conversion of phosphorylase-b to phosphorylase-a by favouring the R-conformation, which is a better substrate for phosphorylase kinase.[27] AMP is known to bind to both the allosteric activation site and the purine nucleoside site.[5] Accordingly, flavopiridols may counteract the effect of AICAR by inhibiting ZMP binding to the purine nucleoside site.

Of the five flavopiridols tested analogues 1 and 4 were the most potent at inhibiting glycogenolysis in a glucose-free medium. In liver, unlike in muscle, phosphorylase-b is catalytically inactive in physiological conditions and only phosphorylase-a is involved in glycogenolysis.[3] Inhibition of glycogenolysis by phosphorylase inhibitors may therefore involve two mechanisms: depletion of phosphorylase-a by dephosphorylation and allosteric inhibition of phosphorylase-a. The inhibition of glycogenolysis by analogues 1 and 4 was greater than could be explained by depletion of phosphorylase-a indicating an important role for allosteric inhibition of phosphorylase-a. There was an apparent lower affinity of the flavopiridols on glycogenolysis as compared with counteraction of phosphorylase activation by AICAR. Since the latter most likely represents binding of the flavopiridols to phosphorylase-b, this difference in affinity can be best explained by the higher affinity of flavopiridols for phosphorylase-b than phosphorylase-a.[20]

The difference in potency between the various flavopiridols on glycogenolysis (1,4 > 2, 3, F) could not be explained by the affinity of the inhibitor for either purified muscle phosphorylase-b or phosphorylase-a, assayed in the direction of glycogen degradation (unpublished results). A possible explanation is that liver phosphorylase shows greater differential selectivity for the flavopiridol derivatives than the muscle

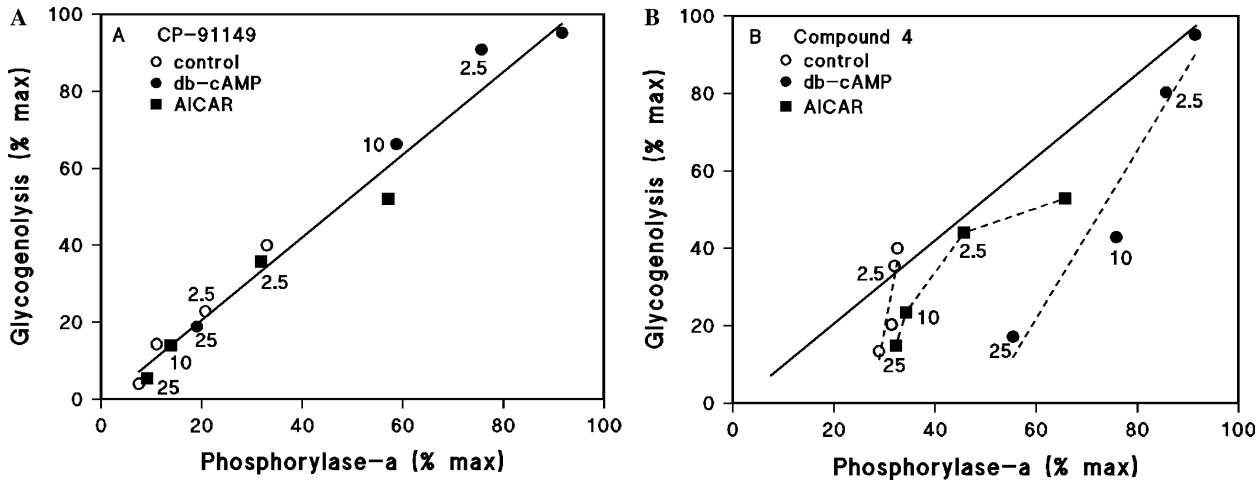


Figure 5. Correlation between glycogenolysis and the amount of phosphorylase-a. Experimental details for determination of glycogenolysis and phosphorylase-a were as in given Figures 3 and 4. Rates of glycogenolysis and phosphorylase-a activity are expressed as percentage of the values in the presence of dibutyryl cAMP without inhibitor (% max). (A) Values for incubations with CP-91149 (2.5–25 μM) and (B) values for incubations with analogue **4** (2.5–25 μM).

isoform. Kasvinsky et al.[29] showed that the endogenous ligands, inosine, FMN and folic acid, inhibited the muscle but not liver isoform of phosphorylase, indicating greater specificity of the purine nucleoside site of the liver isoform. Further work is required to explore the selectivity of the purine nucleoside site of the liver isoform.

This study demonstrates three distinguishing features between the mechanism of action of flavopiridols and indole carboxamides on glycogen metabolism. First, allosteric inhibition of phosphorylase was a significant component of the mechanism of action of flavopiridols but not of the indole carboxamide. Second, the potency of the indole carboxamide was decreased in the presence of dibutyryl-cAMP, whereas that of analogues **1** and **4** was unchanged or slightly increased by dibutyryl-cAMP because of the additional role of enzyme inactivation mediated by binding to phosphorylase-b. Third, the flavopiridols caused negligible stimulation of glycogen synthesis when compared with the indole carboxamide at concentrations that cause comparable inhibition of glycogenolysis, consistent with the lack of depletion of phosphorylase-a in basal conditions.

Two issues have implications in terms of therapeutic use for treatment of Type-2 diabetes. First, flavopiridols are predicted to inhibit glycogenolysis in the postabsorptive state without promoting glycogen storage in the absorptive state, similar to DAB but unlike indole carboxamides. Second, ligands of the purine nucleoside site may enable better differential selectivity between muscle and liver isoforms.

4. Materials and methods

4.1. Materials

CP-91149 was a gift from Prizer Global Research & Development, Groton Laboratories, and DAB was a gift from Novo Nordisk A/S or from Sigma. AICAR and dibutyryl cAMP were obtained from Sigma. Reagents and solvents were obtained from commercial suppliers and used without further purification. Solvents used were of analytical grade. Dichloromethane was distilled from P_2O_5 under Ar atmosphere and kept under Ar and MS 4 Å. Pyridine was first left overnight at stirring with KOH, and then distilled from BaO under N_2 atmosphere and kept under N_2 with MS 4 Å. THF was distilled from Na, with benzophenone as an indicator, under Ar atmosphere.

4.2. General chemistry

Melting points were measured on a Barloworld Scientific-SMP11 apparatus and are given uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 250 MHz spectrometer. Chemical shifts are given in parts per million (δ) relative to TMS and coupling constants are in hertz. Mass spectra were obtained by a Micromass UK Limited Platform LC spectrometer in positive ion spray mode with a flow rate of 10 μL/min. IR- spectra were recorded on a Shimadzu FT-IR 8400S spectrometer.

4.2.1. 1-Methyl-4-(2-hydroxy-3-acetyl-4,6-dimethoxyphenyl)-1,2,3,6-tetrahydropyridine (6). In a three-necked, round-bottomed flask, equipped with a reflux condenser and magnetic stirring bar, under N_2 atmosphere, 1 g (3.8 mmol) of compound **5** was dissolved in 16 ml of dry CH_2Cl_2, and the flask was cooled in an ice bath. To this solution, 3.3 ml (27 mmol) of $BF_3{\cdot}OEt_2$ was added followed by a dropwise addition of 3.6 ml (19 mmol) Ac_2O. The resulted solution was then allowed to stir overnight at rt. Then an aqueous solution of 20% Na_2CO_3 was added until pH 8. The organic phase was separated, the crude product extracted from the aqueous phase with dichloromethane and the combined organic extracts were dried ($MgSO_4$) and evaporated under reduced pressure. The crude product was purified with

SiO_2 column chromatography (eluant: CH_2Cl_2/MeOH/NH_4OH, 90:9:1, v/v/v) and gave **6** as a yellow solid (610 mg, 55% yield). Mp: 119–122 °C; ^1H NMR (250 MHz, $CDCl_3$) δ: 13.90 (s, 1H), 5.94 (s, 1H), 5.53 (m, 1H), 3.88 (s, 3H), 3.81 (s, 3H), 3.10 (m, 2H), 2.67 (t, 2H, $J = 5$ Hz), 2.58 (s, 3H), 2.38 (s, 3H), 2.34 (br m, 2H); ^{13}C NMR (250 MHz, $CDCl_3$) δ: 203.3, 163.3, 162.9, 162.4, 128.6, 125.1, 112.0, 105.8, 85.9, 55.6, 55.4, 54.6, 52.2, 45.8, 33.2, 29.3; MS (ESI): $[M+H]^+ = 292$; FT-IR (KBr pellet): 3430, 2951, 2849, 1631, 1599, 1440, 1270, 1270, 900, 802 cm^{-1}.

4.2.2. 2-Acetyl-3,5-dimethoxy-6-(1-methyl-1,2,3,6-tetrahydropyridin-4-yl)phenyl 2-chlorobenzoate (7a). 2-Chlorobenzoyl chloride (0.65 ml; 5.1 mmol) was added dropwise to a solution of compound **6** (500 mg, 1.7 mmol) and DMAP (20 mg, 0.2 mmol) in 7 ml of dry pyridine, and the solution was stirred for 45 min, under nitrogen atmosphere, at rt. Then, the resulted mixture was poured into a saturated aqueous solution of $NaHCO_3$ (15 ml) and stirred for few minutes. The organic phase was separated, the crude product was extracted from the aqueous phase with dichloromethane and the combined organic extracts were dried and evaporated under reduced pressure. The crude product was purified with SiO_2 column chromatography (eluant: CH_2Cl_2/MeOH, 1:4, v/v) and gave **7a** as a brown solid (533 mg, 73% yield). Mp: 138–142 °C; ^1H NMR (250 MHz, $CDCl_3$) δ: 7.99 (m, 1H), 7.44 (m, 2H), 7.32 (m, 1H), 6.39 (s, 1H), 5.57 (s, 1H), 3.88 (s, 3H), 3.83 (s, 3H), 2.93 (m, 2H), 2.52 (t, 2H, $J = 5$ Hz), 2.47 (s, 3H), 2.34 (br s, 2H), 2.28 (s, 3H); ^{13}C NMR (250 MHz, $CDCl_3$) δ: 199.7, 163.6, 159.7, 157.7, 145.8, 133.8, 132.8, 131.9, 130.9, 129.0, 128.5, 126.6, 125.6, 118.5, 116.8, 92.9, 55.8, 55.7, 54.3, 52.0, 45.5, 31.9, 29.1; MS (ESI): $[M+H^+] = 431$; FT-IR (KBr pellet): 2943, 2778, 1720, 1680, 1606, 1562, 1463, 1328, 1246, 1100, 755 cm^{-1}.

4.2.3. 2-Acetyl-3,5-dimethoxy-6-(1-methyl-1,2,3,6- tetrahydropyridin-4-yl)phenyl 3-chlorobenzoate (7b). As described above for the preparation of compound **7a**, we followed the same procedure using 3-chlorobenzoyl chloride. Product **7b** was isolated as a brown solid in 69% yield (504 mg). Mp: 53–59 °C; ^1H NMR (250 MHz, $CDCl_3$) δ 8.02 (s, 1H), 7.95 (m, 1H), 7.55 (m, 1H), 7.38 (m, 1H), 6.39 (s, 1H), 5.53 (s, 1H), 3.89 (s, 3H), 3.83 (s, 3H), 2.91 (m, 2H), 2.52 (m, 2H), 2.45 (s, 3H), 2.36 (m, 2H), 2.28 (s, 3H); ^{13}C NMR (250 MHz, $CDCl_3$) δ 199.4, 163.9, 159.7, 158.0, 146.2, 134.6, 133.5, 130.9, 130.0, 129.8, 128.7, 128.1, 124.8, 118.1, 116.8, 93.0, 55.9, 55.8, 53.7, 51.5, 44.6, 31.9, 28.2; MS (ESI) *m/z* 431 $[M+H^+]$; IR (KBr) 2940, 2777, 1738, 1680, 1601, 1557, 1460, 1331, 1247, 1103, 755 cm^{-1}.

4.2.4. 2-Acetyl-3,5-dimethoxy-6-(1-methyl-1,2,3,6-tetrahydropyridin-4-yl)phenyl 4-chlorobenzoate (7c). As described above for the preparation of compound **7a**, we followed the same procedure using 4-chlorobenzoyl chloride. Product **7c** was isolated as a brown solid in 64% yield (468 mg). Mp: 89–92 °C; ^1H NMR (250 MHz, $CDCl_3$) δ 7.97 (d, 2H, $J = 7.5$ Hz), 7.37 (d, 2H, $J = 7.5$ Hz), 6.40 (s, 1H), 5.53 (br s, 1H), 3.90 (s, 3H), 3.84 (s, 3H), 2.95 (m, 2H), 2.59 (m, 2H), 2.46 (s, 3H), 2.40 (m, 2H), 2.32 (s, 3H); ^{13}C NMR (250 MHz, $CDCl_3$) δ 199.5, 164.2, 159.7, 157.9, 146.3, 140.0, 131.4, 130.9, 128.9, 127.9, 124.9, 118.1, 116.9, 93.0, 55.9, 55.8, 53.7, 51.6, 44.7, 32.0, 28.3; MS (ESI) *m/z* 431 $[M+H^+]$; IR (KBr) 2940, 2779, 1728, 1675, 1600, 1559, 1460, 1330, 1247, 1100, 755 cm^{-1}.

4.2.5. 2-Acetyl-3,5-dimethoxy-6-(1-methyl-1,2,3,6-tetrahydropyridin-4-yl)phenyl benzoate (7d). As described above for the preparation of compound **7a**, we followed the same procedure using benzoyl chloride. Product **7d** was isolated as a brown solid in 75% yield (505 mg). Mp: 156–160 °C; ^1H NMR (250 MHz, $CDCl_3$) δ 8.07 (d, 2H, $J = 7.5$ Hz), 7.59 (tr, 1H, $J = 7.5$ Hz), 7.45 (tr, 2H, $J = 7.5$ Hz), 6.39 (s, 1H), 5.54 (br s, 1H), 3.89 (s, 3H), 3.83 (s, 3H), 2.82 (m, 2H), 2.48 (m, 2H), 2.46 (s, 3H), 2.37 (m, 2H), 2.24 (s, 3H); ^{13}C NMR (250 MHz, $CDCl_3$) δ 199.7, 165.1, 169.6, 157.6, 146.4, 133.4, 130.1, 129.2, 128.6, 128.4, 125.6, 118.4, 117.3, 92.9, 55.9, 55.8, 54.2, 52.0, 45.3, 32.0, 28.8; MS (ESI) *m/z* 396 $[M+H^+]$; IR (KBr) 2938, 2777, 1728, 1677, 1603, 1562, 1464, 1333, 1252, 1108, 700 cm^{-1}.

4.2.6. 2-(2-Chlorophenyl)-5,7-dimethoxy-8-(1-methyl-1,2,3,6-tetrahydropyridin-4-yl)-4*H*-benzopyran-4-one (8a). Compound **7a** (800 mg, 1.9 mmol) was dissolved in dry THF (20 ml) and stirred under nitrogen at rt. Then 170 mg NaH (60% in paraffin oil, 4.2 mmol) was added and the mixture was stirred at 60 °C for 1 h. Then the mixture was left to cool down and treated with MeOH to quench the excess of NaH. The solvents were removed under vacuum. The crude product was dissolved in 12 ml of glacial acetic acid, 100 µl concentrated H_2SO_4 was added and the solution was stirred at 100 °C for 1 h. Solvents were removed under reduced pressure. The residue was dissolved in water and the pH was adjusted to 8 with aqueous 15% Na_2CO_3 solution. The crude product was extracted from the aqueous phase with dichloromethane and the organic extracts were combined, dried and evaporated under reduced pressure. The crude product was purified with SiO_2 column chromatography (eluant: CH_2Cl_2/MeOH/NH_4OH, 90:9:1, v/v/v) and afforded **8a** as a yellow solid (430 mg, yield 55%); Mp: 145–152 °C; ^1H NMR (250 MHz, $CDCl_3$) δ: 7.60 (m, 1H), 7.48 (m, 1H), 7.38 (m, 2H), 6.56 (s, 1H), 6.42 (s, 1H), 5.63 (br s, 1H), 4.00 (s, 3H), 3.90 (s, 3H), 3.10 (m, 2H), 2.64 (t, 2H, $J = 7.5$ Hz), 2.38 (br s, 5H); ^{13}C NMR (250 MHz, $CDCl_3$) δ: 177.7, 160.9, 160.3, 159.7, 156.1, 132.6, 131.6, 131.4, 130.8, 130.6, 127.4, 126.9, 126.3, 113.8, 112.4, 108.6, 91.7, 56.4, 55.9, 54.5, 52.1, 45.6, 29.5; MS (ESI): $[M+H^+] = 413$; FT-IR (KBr pellet): 2960, 2798, 1649, 1596, 1479, 1380, 1331, 1210, 1122, 765 cm^{-1}.

4.2.7. 2-(3-Chlorophenyl)-5,7-dimethoxy-8-(1-methyl-1,2,3,6-tetrahydropyridin-4-yl)-4*H*-benzopyran-4-one (8b). As described above for the preparation of **8a**, we followed the same procedure using **7b**. Product **8b** was isolated as a dark yellow solid in 45% yield (353 mg). Mp: 170–178 °C; ^1H NMR (250 MHz, $CDCl_3$) δ 7.89 (m, 1H), 7.67 (d, 1H, $J = 7.5$ Hz), 7.41 (m, 2H), 6.63

(s, 1H), 6.40 (s, 1H), 5.72 (br s, 1H), 3.98 (s, 3H), 3.90 (s, 3H), 3.21 (m, 2H), 2.74 (tr, 2H, $J = 5$ Hz), 2.45 (br s, 5H); ^{13}C NMR (250 MHz, $CDCl_3$) δ 177.8, 161.0, 159.9, 158.6, 155.3, 135.1, 133.3, 130.9, 130.1, 127.9, 126.6, 126.1, 123.7, 112.3, 108.6, 108.3, 91.6, 56.3, 55.8, 54.8, 52.4, 45.9, 29.8; MS (ESI) *m/z* 413 [M+H$^+$]; IR (KBr): 2936, 2790, 1656, 1593, 1475, 1376, 1330, 1120, 1115, 801, 699 cm^{-1}.

4.2.8. 2-(4-Chlorophenyl)-5,7-dimethoxy-8-(1-methyl-1,2,3,6-tetrahydropyridin-4-yl)-4*H*-benzopyran-4-one (8c). As described above for the preparation of **8a**, we followed the same procedure using **7c**. Product **8c** was isolated as a yellow solid in 51% yield (400 mg). Mp: 182–186 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.77 (d, 2H, $J = 5$ Hz), 7.45 (d, 2H, $J = 5$ Hz), 6.61 (s, 1H), 6.41 (s, 1H), 5.69 (br s, 1H), 3.99 (s, 3H), 3.90 (s, 3H), 3.17 (m, 2H), 2.71 (m, 2H), 2.45 (s, 3H), 2.43 (br m, 2H); ^{13}C NMR (250 MHz, $CDCl_3$) δ 177.9, 160.9, 159.9, 159.3, 155.5, 137.2, 130.1, 129.2, 127.9, 127.1, 126.5, 112.5, 108.6, 108.0, 91.6, 56.3, 55.9, 54.9, 52.5, 45.9, 29.8; MS (ESI) *m/z* 413 [M+H$^+$]; IR (KBr): 2935, 2799, 1651, 1594, 1480, 1364, 1324, 1278, 1126, 820 cm^{-1}.

4.2.9. 2-Phenyl-5,7-dimethoxy-8-(1-methyl-1,2,3,6-tetrahydropyiridin-4-yl)-4*H*-benzopyran-4-one (8d). As described above for the preparation of **8a**, we followed the same procedure using **7d**. Product **8d** was isolated as a yellow solid in 44% yield (316 mg). Mp: 184–190 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.80 (br s, 2H,), 7.45 (br s, 3H), 6.62 (s, 1H), 6.38 (s, 1H), 5.58 (br s, 1H), 3.96 (s, 3H), 3.87 (s, 3H), 3.14 (m, 2H), 2.68 (m, 2H), 2.42 (br s, 5H); ^{13}C NMR (250 MHz, $CDCl_3$) δ 178.0, 160.7, 160.3, 159.8, 155.5, 131.5, 130.9, 128.7, 127.7, 126.4, 125.7, 112.2, 108.5, 107.8, 91.4, 56.5, 55.7, 54.8, 52.4, 45.9, 29.7; MS (ESI) *m/z* 378 [M+H$^+$]; IR (KBr): 2930, 2795, 1642, 1593, 1465, 1380, 1332, 1116, 780, 679 cm^{-1}.

4.2.10. 2-(2-Chlorophenyl)-5,7-dihydroxy-8-(1-methyl-1,2,3,6-tetrahydropyridin-4-yl)-4*H*-benzopyran-4-one (1). Compound **8a** (25 mg, 0,06 mmol) was placed in a sealed tube together with hydrochloric pyridine (300 mg, 2.6 mmol) and heated to 180 °C. The solution then was stirred for 2 h. The mixture was left to cool down and a saturated aqueous solution of $NaHCO_3$ was added. The crude product was extracted from the aqueous phase with dichloromethane/methanol (9:1, v/v) and the organic extracts were combined, dried and evaporated under reduced pressure. The crude product was purified with SiO_2 column chromatography (eluant: CH_2Cl_2/MeOH/NH_4OH, 90:9:1, v/v/v) and afforded **1** as a green solid (13 mg, yield 56%); Mp: 205–211 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.39–7.58 (m, 4H), 6.51 (s, 1H), 6.28 (s, 1H), 5.74 (br s, 1H), 3.25 (br s, 2H), 2.82 (m, 2H), 2.57 (br s, 5H) [data in agreement with the literature[3]]; ^{13}C NMR (250 MHz, $CDCl_3$) δ 182.3, 163.0, 161.4, 160.7, 154.8, 132.6, 131.4, 131.9, 130.9, 130.6, 128.0, 127.4, 127.1, 110.9, 108.2, 104.7, 99.8, 54.1, 51.9, 44.8, 29.2; MS (ESI) *m/z* 385 [M+H$^+$]; IR (KBr): 3440, 2921, 1655, 1577, 1419, 1369, 1275, 1186, 1101, 760 cm^{-1}.

4.3. Supplementary data

4.3.1. 1-Methyl-4-(2,4,6-trimethoxyphenyl)-1,2,3,6-tetrahydropyridine (5). ^{13}C NMR (250 MHz, $CDCl_3$) δ 159.8, 158.2, 129.1, 124.4, 113.2, 90.6, 55.7, 55.1, 54.7, 52.3, 45.9, 29.8; IR (KBr): 2879, 2783, 1604, 1460, 1411, 1203, 1132, 1037, 980, 810 cm^{-1}.

4.3.2. 2-(3-Chlorophenyl)-5,7-dihydroxy-8-(1-methyl-1,2,3,6-tetrahydropyridin-4-yl)-4*H*-benzopyran-4-one (2). ^{13}C NMR (250 MHz, $CDCl_3$) δ 182.4, 161.5, 161.3, 161.2, 154.1, 135.2, 133.0, 131.5, 131.4, 128.2, 128.0, 126.1, 124.0, 108.0, 105.8, 104.7, 99.9, 54.5, 52.3, 45.1, 29.5; IR (KBr): 3440, 2924, 1653, 1581, 1423, 1375, 1275, 1186, 1105, 760 cm^{-1}.

4.3.3. 2-(4-Chlorophenyl)-5,7-dihydroxy-8-(1-methyl-1,2,3,6-tetrahydropyridin-4-yl)-4*H*-benzopyran-4-one (3). ^{13}C NMR (250 MHz, $CDCl_3$) δ 183.8, 164.0, 163.3, 161.7, 155.5, 139.1, 130.9, 130.4, 129.3, 128.7, 126.2, 113.1, 109.5, 105.9, 100.2, 54.8, 52.8, 45.2, 29.3; IR (KBr): 3440, 2921, 1581, 1419, 1375, 1275, 1188, 1103, 831 cm^{-1}.

4.3.4. 2-Phenyl-5,7-dihydroxy-8-(1-methyl-1,2,3,6-tetrahydropyridin-4-yl)-4*H*-benzopyran-4-one (4). ^{13}C NMR (250 MHz, $CDCl_3$) δ 182.7, 163.6, 161.3, 160.6, 154.3, 131.7, 131.2, 129.0, 128.1, 127.6, 125.9, 108.1, 105.4, 104.7, 99.7, 54.4, 52.6, 45.0, 29.4; IR (KBr): 3440, 2943, 2775, 1641, 1583, 1423, 1279, 1184, 1101, 773, 692 cm^{-1}.

4.3.5. 2,6-Di(1,2,3,6-tetrahydropyridin-4-yl)-3,5-dimethoxy phenol (10). ^{1}H NMR (250 MHz, $CDCl_3$) δ 6.04 (s, 1H), 5.54 (br s, 2H), 3.76 (s, 6H), 3.08 (m, 4H), 2.64 (t, 4H, $J = 5$ Hz), 2.38 (br s, 10H); MS (ESI) *m/z* 345 [M+H$^+$], 173 [(M+2H$^+$)/2]

4.4. Phosphorylase kinetics

Rabbit glycogen phosphorylase-b was isolated, purified, recrystallised, and assayed as described.[34] Kinetic experiments with flavopiridol and analogues were performed in the direction of glycogen synthesis in the presence of constant concentrations of glycogen (0.2% w/v), AMP (1 mM), and various concentrations of Glc-1-P (1.5–20 mM) and inhibitors (0.25–10 μM), in 30 mM imidazole/HCl buffer (pH 6.8), 60 mM KCl, 0.6 mM EDTA, 0.6 mM dithiothreitol, and 0.1% DMSO.

4.5. Hepatocyte isolation and culture

Hepatocytes were isolated by collagenase perfusion of the liver[10] of male Wistar rats (body wt 200–250 g) obtained from B&K, Hull, UK. All experiments were conducted in accordance with EC Council Directive (86/609/EEC). The hepatocytes were suspended in MEM supplemented with 5% new born calf serum and plated in 24-well plates for determination of glycogen synthesis and phosphorylase-a activity and in gelatine coated 96-well plates for determination of glycogenolysis. After cell attachment (approximately 4 h) the medium was replaced by serum-free MEM containing 10 nM

dexamethasone, 100 nM insulin and 25 mM glucose for subsequent determination of glycogenolysis and activity of phosphorylase-a and in medium containing 10 nM dexamethasone and 5 mM glucose for subsequent determination of glycogen synthesis.

4.6. Glycogenolysis and phosphorylase-a activity

Hepatocyte monolayers in 96-well plates were pre-cultured for 16 h in MEM containing 100 nM insulin, 25 mM glucose and [U–^{14}C]glucose (2 μCi/ml). They were then washed three times with 150 mM NaCl and incubated for 3 h in glucose-free DMEM (Invitrogen, Life Technologies) containing the inhibitor concentrations and other additions indicated. On termination of the incubation the medium was collected for determination of glucose spectrometrically by coupling to hexokinase and glucose 6-phosphate dehydrogenase[17] and the cells were extracted in 0.1 M NaOH and radiolabelled glycogen was determined by ethanol precipitation.[17] Glycogenolysis was determined from the fractional decrease in radiolabelled glycogen during the 3 h incubation. For determination of phosphorylase-a parallel incubations were performed on hepatocytes in 24-well plates incubated under similar conditions as for glycogenolysis except for omission of the radiolabelled glucose. On termination of the incubations, the plates were snap-frozen in liquid nitrogen and the activity of phosphorylase-a was determined spectrometrically by coupling to phosphoglucomutase and glucose 6-phosphate dehydrogenase as described previously.[10] Activities are expressed as m units/mg cell protein.

4.7. Glycogen synthesis

Hepatocyte monolayers in 24-well plates were pre-cultured for 16 h in MEM containing 5 mM glucose. They were then incubated for 3 h in fresh MEM containing 15 mM glucose and [U–^{14}C]glucose (2 μCi/ml) and other additions as indicated. On termination of the incubations the hepatocytes were washed in 150 mM NaCl and extracted in 0.1 M NaOH. Radiolabelled glycogen was determined as previously described[10] and rates of glycogen synthesis are expressed as nmol of glucose incorporated into glycogen/3 h mg^{-1} cell protein.

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