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CJ-21,164, a New D-Glucose-6-phosphate Phosphohydrolase Inhibitor Produced by a Fungus *Chloridium* sp.

YOON-JEONG KIM*, HIROYUKI NISHIDA, CHANG-HONG PANG, TOSHIYUKI SAITO, SHINICHI SAKEMI, HIROKO TONAI-KACHI, NOBUJI YOSHIKAWA, MARIA A. VANVOLKENBURG†, JANICE C. PARKER† and YASUHIRO KOJIMA

Exploratory Medicinal Sciences, PGRD, Nagoya Laboratories, Pfizer Pharmaceuticals Inc., 5-gochi, Taketoyo-cho, Chita-gun, Aichi 470-2393, Japan
† PGRD, Groton Laboratories, Pfizer Inc., Eastern Point Road, Groton, CT 06340, USA

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A new D-glucose-6-phosphate phosphohydrolase (G6Pase) inhibitor, CJ-21,164 (**I**) was isolated from the fermentation broth of the fungus *Chloridium* sp. CL48903. The structure was elucidated to be a novel tetramer of the salicylic acid derivatives by spectroscopic analyses. Compound **I** inhibited G6Pase in rat liver microsomes with an IC_{50} of 1.6 μM. Glucose output from hepatocytes isolated from rat liver was inhibited when **I** was present in the incubation medium, consistent with the role of **I** as a G6Pase inhibitor.

D-Glucose-6-phosphate phosphohydrolase (G6Pase, E.C. 3.1.3.9) is a key regulatory enzyme in hepatic metabolisms. The two metabolic pathways by which the liver can produce glucose are gluconeogenesis and glycogenolysis. G6Pase catalyzes the final step in both pathways. Excessive hepatic glucose output is a major cause of the fasting hyperglycemia that characterizes diabetes[1]. Since inhibition of G6Pase reduces hepatic glucose output *via* both pathways, it ought to lower blood glucose levels in diabetic subjects.

This hypothesis has been tested using inhibitors of G6Pase[2]. Glucose output was effectively lowered in rat livers perfused with 3-mercaptopicolinic acid, an inhibitor of G6Pase. However, this agent does not target G6Pase specifically, as it also inhibited phosphoenolpyruvate carboxykinase activity[3]. More recently, a series of synthetic derivatives of chlorogenic acid have been identified as selective inhibitors for G6Pase[4], some of which have been shown to reduce blood glucose levels when administered intravenously to normal rats[5] and to improve glucose tolerance in diabetic *ob/ob* mice[6]. However, for reasons of poor potency and bioavailability, none of these compounds has proven suitable for development as a treatment for diabetes in human patients.

Therefore, we set out to identify structurally novel inhibitors of rat hepatic endoplasmic reticulum G6Pase using microsomal membranes to screen microbial extracts. As a result, we found that the fungus *Chloridium* sp. CL48903 produces CJ-21,164 (**I**), which has potent activity against G6Pase. This paper deals with the fermentation, isolation, structural elucidation, and biological activity of **I**.

Results

Isolation

The fermentation broth (1 liter) was filtered after the addition of an equal volume of ethanol. The filtrate was concentrated to an aqueous solution and then extracted twice with 500 ml of ethylacetate. The organic layer was dried over anhydrous Na_2SO_4 and concentrated to dryness. The oily residue was applied onto a column of Sephadex LH-20 (35×850 mm, Amersham Pharmacia Biotech) and eluted with methanol. The active fractions were concentrated and then subjected to a preparative HPLC on an ODS column (YMC-pack ODS AM-343, 20×250 mm,

* Corresponding author: Yoon-Jeong.Kim@japan.pfizer.com

YMC Co. Ltd.). The column was then eluted with acetonitrile - 0.05% trifluoroacetic acid in H_2O (85 : 15) at a flow rate of 4 ml/minute to yield 23.5 mg of **I** as a white powder.

Physico-chemical Properties

Physico-chemical properties of **I** are summarized in Table 1. Compound **I** is soluble in most organic solvents but not in water or *n*-hexane. The molecular formula $C_{38}H_{37}ClO_{14}$ was determined on the basis of negative HRFAB-MS, and the numbers of carbons and hydrogens were confirmed based on the NMR spectra. The M+2 signal in the FAB-MS (about 40% of the molecular ion) supported the presence of a chlorine atom in the molecule. The value of the optical rotation suggested that the molecule has (an) asymmetric center(s). Compound **I** exhibited intense electronic transitions in MeOH at λ_{max} nm (ε) 215 (78,800), 252 (29,900), 284 (12,700) and 315 (sh), which implies a conjugated aromatic system composed of a hydroxy benzoic acid moiety. The IR spectra showed absorption bands due to hydroxyl groups between 3500 and 3200 cm^{-1}, and carbonyl groups around 1700 cm^{-1}.

Structural Elucidation

The structure of **I** was elucidated through HMBC, phase-sensitive ROESY, and FAB-MS analyses. The HMBC gave

Fig. 1. Structure of CJ-21,164 (**I**).

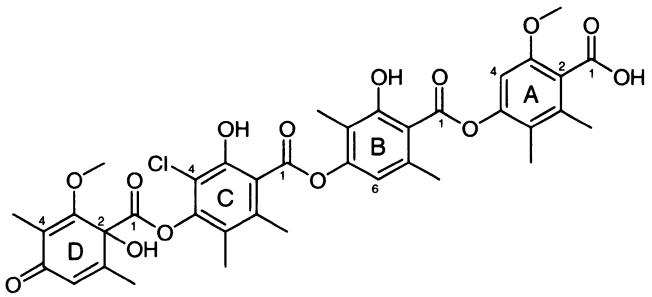
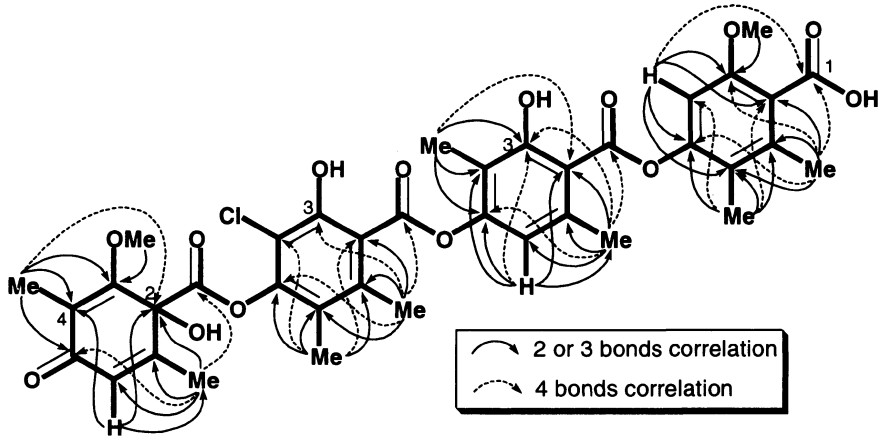


Table 1. Physico-chemical properties of CJ-21,164 (**I**).

Appearance	White powder
Molecular weight	753.14
Molecular formula	$C_{38}H_{37}ClO_{14}$
HRFAB-MS (*m/z*)	(negative)
Found:	751.1782
Calcd. for:	$C_{38}H_{36}ClO_{14}$ (751.1794)
$[\alpha]_D$ (24°C, MeOH)	+63.8° (*c* 1.00)
UV λ_{max} (MeOH) nm (ε)	215 (78,800), 252 (29,900) 284 (12,700), 315 (sh)
IR ν_{max} (KBr) cm^{-1}	3700-2500, 3408, 3250 (sh), 2937, 1760, 1725 (sh), 1676, 1618, 1583, 1445, 1385, 1348, 1296, 1258, 1190, 1140, 1084, 959, 881, 800, 723
LRFAB-MS (*m/z*)	(matrix: DTT/TG12 [a])
Positive:	753/755 (5:2), 557/559, 393/395, 363/365, 359/361, 199/201, 195, 179, 168, 165
Negative:	751/753 (5:2), 557/559, 409/411, 361/363, 359/361, 197/199, 195, 181, 163

[a] 1: 2 mixture of dithiothreitol and α-thioglycerol (Tokyo Kasei Kogyo Co., Ltd., #S0437).

Fig. 2. Summary of ^{13}C-^{1}H long-range couplings obtained by HMBC experiment in acetone-d_6.



many clear 4-bond correlations, although these signals were weaker than those of the 2- or 3-bond couplings (Fig. 2). This allowed us to establish that the compound consists of four connected benzoic acid units (units A, B, C and D).

The assignments of the chemical shifts are summarized in Table 2. In the ^{13}C NMR spectrum, the D-4 carbon was almost unobservable, probably because of its signal-broadening feature. However, through the HMBC, the existence of the D-4 carbon was extrapolated by the presence of cross peaks for D-4-Me and D-6-H. Among the 4-bond correlations, cross peaks for the two carbonyl groups (D-1, D-5) and D-7-Me were observed when we ran HMBC, focusing on the smaller coupling (4~5 Hz) in acetone-d_6.

Carbons attached to hydroxyl groups (carbons A-1, B-3, C-3 and D-2) were specified by a high field shift of ^{13}C NMR signals, which occurred when the hydroxyl groups were replaced by deuteroxyl groups. After the ^{13}C NMR

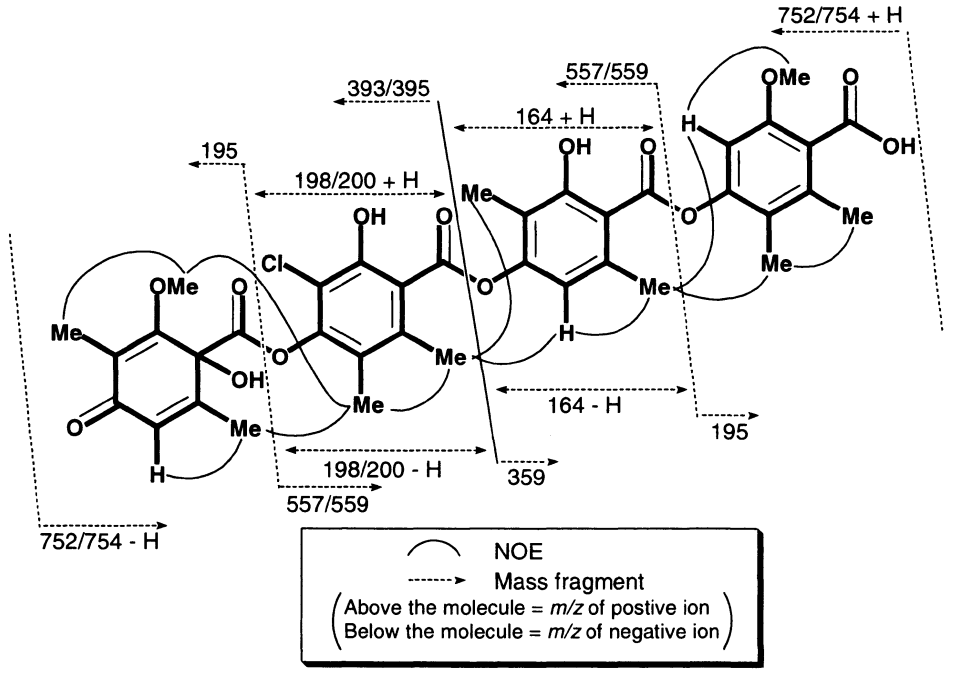
Table 2. ^{13}C and ^{1}H NMR assignment for CJ-21,164 (**I**) in acetone-d_6 and in methanol-d_4.

	acetone-d_6		methanol-d_4	
Atom #	^{13}C (δ, ppm)	^{1}H (δ, ppm)[a]	^{13}C (δ, ppm)	^{1}H (δ, ppm)
A-1	169.6 (s)		172.8 (s)	
A-2	125.5 (s)		126.0 (s)	
A-3	156.1 (s)		156.7 (s)	
A-3-OMe	56.9 (q)	3.77 (3H, s)	57.4 (q)	3.80 (3H, s)
A-4	105.0 (d)	6.94 (1H, s)	105.4 (d)	6.80 (1H, s)
A-5	150.8 (s)		151.8 (s)[b]	
A-6	122.4 (s)		123.2 (s)	
A-6-Me	12.9 (q)	2.06 (3H, s)	13.2 (q)	2.08 (3H, s)
A-7	136.8 (s)		137.5 (s)	
A-7-Me	17.5 (q)	2.25 (3H, s)	17.9 (q)	2.28 (3H, s)
B-1	171.6 (s)		172.1 (s)	
B-2	111.0 (s)		112.1 (s)	
B-3	164.4 (s)		164.6 (s)	
B-4	118.5 (s)		119.3 (s)	
B-4-Me	9.9 (q)	2.122 (3H, s)	10.2 (q)	2.17 (3H, s)
B-5	154.9 (s)		155.6 (s)	
B-6	118.2 (d)	6.80 (1H, s)	118.7 (d)	6.73 (1H, s)
B-7	141.4 (s)		141.7 (s)	
B-7-Me	24.7 (q)	2.68 (3H, s)	24.8 (q)	2.69 (3H, s)
C-1	166.8 (s)		167.8 (s)	
C-2	121.6 (s)		123.7 (s)	
C-3	151.7 (s)		151.7 (s)[b]	
C-4	114.0 (s)		114.6 (s)	
C-5	148.4 (s)		148.6 (s)	
C-6	124.5 (s)		124.8 (s)	
C-6-Me	13.0 (q)	2.03 (3H, s)	13.4 (q)	2.04 (3H, s)
C-7	137.0 (s)		136.6 (s)	
C-7-Me	18.1 (q)	2.40 (3H, s)	18.1 (q)	2.36 (3H, s)
D-1	168.7 (s)		169.0 (s)	
D-2	78.3 (s)		78.9 (s)	
D-3	167.8 (s)		169.7 (s)	
D-3-OMe	62.9 (q)	4.05 (3H, s)	63.3 (q)	4.08 (3H, s)
D-4	121.1 (s br)		121.5 (s br)	
D-4-Me	9.4 (q)	1.90 (3H, s)	9.7 (q)	1.94 (3H)
D-5	188.1 (s)		190.5 (s)	
D-6	128.8 (d)	6.21 (1H, q, J = 1.5 Hz)	129.1 (d)	6.24 (1H, q, J = 1.5 Hz)
D-7	152.1 (s)		154.5 (s)	
D-7-Me	18.6 (q)	2.120 (3H, br s)	19.0 (q)	2.15 (3H, d, J = 1.5 Hz)

[a] Three of four hydroxyl protons were observed as broad signals at δ 11.57, 9.81 (very broad) and 5.97 ppm in acetone-d_6.

[b] Exchangeable assignments.

Fig. 3. NOEs by phase-sensitive ROESY (in methanol-d_4) and FAB-MS fragments of CJ-21,164.



was taken in acetone-d_6, the NMR-sample tube contents were repeatedly dried under a flow of nitrogen gas and dissolved three times in 100 μl of methanol-d_4. The resulting dried deuteroxylized sample was dissolved in the same amount of acetone-d_6 as before, and its ^{13}C NMR was taken again. The chemical shifts of the deuteroxylized sample were compared to those of the unaltered one. As a result, the chemical shifts of the carbons at A-1, B-3, C-3 and D-2 were upper field shifted [from 169.55 to 169.46 (Δ 0.09), 164.37 to 164.15 (Δ 0.22), 151.73 to 151.66 (Δ 0.07) and 78.28 to 78.21 (Δ 0.07) ppm, respectively], while the other signals shifted around 0.02 ppm or less.

An alternative structure could be possible for unit D from the HMBC data, in which the positions of the D-3-OMe and D-5 carbonyl groups were exchanged. However, this alternative structure was eliminated because of the clear NOE between the D-3-OMe and C-6-Me (Fig. 3) but a lack of NOE between the D-3-OMe and D-6-H. The chemical shift of the D-5 carbonyl group (δ 188.1 ppm in acetone-d_6) also supported the position of the carbonyl group being at D-5 rather than at D-3[7].

The composition of each benzoic acid unit, including the placement of the chlorine atom, was also supported by the corresponding FAB-MS fragments: *m/z* 195 for units A and D; *m/z* 164+/−H for unit B; and *m/z* 198/200+/−H for unit C, which contained the chlorine atom.

The sequencing of these benzoic acid units was determined by analysis of NOESY and FAB-MS (Fig. 3). The phase-sensitive ROESY spectrum in methanol-d_4 showed cross-peaks between A-4-H/A-6-Me and B-7-Me, B-4-Me/B-6-H and C-7-Me, and C-6-Me and D-3-OMe/D-7-Me. Thus, units A through D are connected *via* ester bonds. The assignments of these connections were also made by FAB-MS analysis, which supported the proposed structure by showing a sequential fragmentation of the benzoic acid units. The positive mode showed *m/z* 752/754+H (units A-B-C-D), *m/z* 557/559 (units B-C-D), *m/z* 393/365 (units C-D), and *m/z* 195 (unit D); while the negative mode showed *m/z* 752/754−H (units A-B-C-D), *m/z* 557/559 (units A-B-C), *m/z* 359 (units A-B), and *m/z* 195 (unit A), as shown in Fig. 3. The structure of CJ-21,164 was thus elucidated as shown in Fig. 1.

Biological Activities

The biological activities of **I** are summarized in Table 3. It dose-dependently inhibited G6Pase, with an IC_{50} value of 1.6 μM. At a concentration of 133 μM, this compound inhibited the rate of glucose output stimulated with 25 nM glucagon by 81%. Hepatocytes incubated with **I** did not

Table 3. Biological activities of CJ-21,164 (**I**).

Concentration (μM)	G6Pase inhibition (%)	Glucose output inhibition (%)	Cytotoxicity (%)
1.6	50	NT	NT
16	104	NT	NT
133	102	81	10

NT: not tested

Inhibition of enzyme activity and inhibition of glucose output from isolated rat hepatocytes were measured as described in the experimental section. Cytotoxicity was assessed in isolated rat heptocytes by trypan blue exclusion as described in the experimental section.

show significant cytotoxicity at these concentrations, suggesting that the reduction in glucose output was not a consequence of cytotoxicity.

Discussion

We have isolated a new G6Pase inhibitor, consisting of a tetramer of salicylic acid derivatives, and demonstrated its glucose-output inhibition in rat hepatocytes.

A number of salicylic acid related compounds have been found to exhibit a wide variety of biological activities, the most famous being aspirin [2-(acetyloxy)benzoic acid], which is widely used for its analgesic, antipyretic and anti-inflammatory properties. A group of salicylic acid derivatives having aliphatic side chains, such as anacardic acid[8], was reported to possess antineoplastic, antibacterial, molluscicidal, and nematocidal activities. According to SAKEMI *et al.*, thielavins[9], trimers of salicylic acid derivatives, also showed activities against G6Pase. These were reported as prostagladin biosynthesis inhibitors[10], while the dimer of salicylic acid derivatives and the anacardic acid related compounds did not inhibit the enzyme.

The trimer (thielavins) and tetrameter (**I**) of salicylic acid derivatives, unlike the dimers and monomers, may be conforming to the shape and/or size of the active site of the enzyme due to their 3-dimensional mobility, which would account for their G6Pase inhibitory activities.

Compound **I** was also functionally active in an isolated cell system, inhibiting the output of glucose from isolated rat hepatocytes. This suggests that this class of compounds may have therapeutic potential as anti-diabetic agents, since hepatic glucose output is elevated in diabetic patients[1], furthermore, G6Pase inhibitors that reduce glucose output from hepatocytes have been shown to be effective at lowering blood-glucose levels in animal models of diabetes[6]. However, the comparatively low potency of **I** precluded testing for *in vivo* effects. Further investigation of the possible therapeutic benefits of agents of this class will depend upon the development of more potent analogues with appropriate pharmacokinetic and pharmacodynamic properties.

Experimental

General

The IR spectrum was recorded on a Shimadzu IR-470 spectrometer. The UV spectra were obtained on a JASCO Ubest-30. Optical rotations were determined on a JASCO DIP-370 with a 5 cm cell. The FAB-MS and HRFAB-MS were measured on a JEOL JMS-700 spectrometer. The NMR spectra were obtained on a JEOL JNM-Lambda270 in acetone-d_6 (δ 2.00 and 30.3 ppm for the ^{1}H and ^{13}C references) and in methanol-d_4 (δ 3.30 and 49.8 ppm for the ^{1}H and ^{13}C references). Field gradient-HMBC and phase-sensitive ROESY data were also obtained on a Bruker AVANCE 600 NMR Spectrometer in acetone-d_6 and in methanol-d_4.

Producing Microorganism

The producing strain, the fungus *Chloridium* sp. CL48903, was obtained from the Sichuan Industrial Institute of Antibiotics, China.

Fermentation

Chloridium sp. CL48903 was maintained on an agar slant of Potato Dextrose Agar (Difco). The cell suspension was inoculated into a 500 ml Erlenmeyer flask containing

100 ml of a seed medium consisting of Potato Dextrose Broth (Difco) 2.4%, yeast extract (Difco) 0.5%, and Bacto-Agar (Difco) 0.1%. The flask was incubated at 27°C on a rotary shaker at 250 rpm for 4 days. The seed culture (100 ml) was transferred to ten 500 ml Erlenmeyer flasks containing 100 ml of a production medium consisting of sucrose 8%, corn flour 5%, and yeast extract 0.1% (pH 6.1 before sterilization). Fermentation was carried out at 27°C on a rotary shaker at 250 rpm for 18 days.

Preparation of Rat Liver Microsomes

Sprague-Dawley rats (male, 200~220 g) were fasted overnight, anaesthetized with ether, and had their livers excised, which were immediately washed with saline. The weight was recorded for each liver. The livers were chopped up and homogenized for 30 seconds (Polytron) in a homogenization buffer (250 mM sucrose, 25 mM HEPES, 2.5 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4). The homogenate was centrifuged for 10 minutes at 4°C (12,000 *g*), and then the resulting supernatant was ultracentrifuged for 60 minutes at 4°C (100,000 *g*). The resulting microsomal pellet was resuspended in the homogenization buffer at a concentration of 1 g (wet weight) of liver per ml and stored in aliquot parts at −80°C. The microsomal preparation was diluted 1 : 60 with the glucose-6-phosphate-free assay buffer described below and was used for this screening.

G6Pase Activity

The activity of the G6Pase was measured based on the colorimetric reaction of inorganic phosphate. The enzyme reaction was initiated by adding 20 μl of microsome suspension (see above) to the reaction mixture, which contained 10 μl of test sample and 70 μl of assay buffer (50 mM HEPES, 100 mM KCl, 2.5 mM ethyleneglycol bis-2-aminoethylether-tetraacetic acid, 2.5 mM $MgCl_2$, 1 mM dithiothreitol and 1.43 mM glucose-6-phosphate, pH 7.2 adjusted by KOH). After 15 minutes of incubation at room temperature, 150 μl of reagent (one part 34 mM ammonium molybdate in HCl to three parts 1.3 mM malachite green) was added, and the absorbance at 690 nm was measured. The percentage of inhibition for G6Pase activity was calculated as follows:

$$\text{Inhibition (\%)} = 100 \times [\{A_{690}\text{ (control)} - A_{690}\text{ (sample)}\} / \{A_{690}\text{ (control)} - A_{690}\text{ (background)}\}],$$

where A_{690} was the absorbance at 690 nm.

Glucose Output Activity in Rat Hepatocytes

Hepatocytes were prepared from the livers of fed male Sprague-Dawley rats by collagenase digestion[11] and incubated as described previously[6]. The cells were suspended in Krebs-Henseleit bicarbonate (KRB) buffer (pH 7.4) containing 2.5 mM $CaCl_2$ and 1% gelatin (w/v). The hepatocyte suspensions (6×10^6 cells/ml) were incubated in 25 ml flasks in a 37°C shaking water bath and continuously gassed ($95\%O_2+5\%CO_2$). The cells were then pre-incubated for five minutes in two groups, one in 0.1% DMSO with the test compound and the other in 0.1% DMSO alone. Then, each group was further subdivided by adding either 20 μl of KRB or 25 nM glucagon. Finally all four groups were incubated for 30 minutes. Aliquot parts of cell suspension were removed and centrifuged. The glucose content of the resulting supernatant buffer was measured using a CCX Spectrum Systems Autoanalyzer (Abbott, North Chicago, IL), and the change in glucose concentration (ΔGlc) for each condition was calculated. The percentage inhibition of glucose output was calculated as follows:

$$\text{Inhibition (\%)} = [\{\Delta\text{Glc(total)} - \Delta\text{Glc(sample)}\} / \{\Delta\text{Glc(total)} - \Delta\text{Glc(basal)}\}] \times 100,$$

where ΔGlc (total) was glucose output by cells treated with glucagon, but without test compound; ΔGlc (sample) was glucose output by cells treated with both glucagon and test compound and ΔGlc (basal) was glucose output by untreated cells.

Cytotoxicity

Cytotoxicity was assessed in hepatocytes that had been incubated with **I** to measure glucose output. After incubation, an aliquot part of cell suspension was mixed with an 8-fold volume of trypan blue (0.4% (w/v) in saline) and counted on a haemocytometer. Cells that did not absorb the trypan blue were scored as viable, while cells that stained blue were considered dead. The results were expressed as a percentage of blue (dead) cells to total cells. No correction was made for the percentage of cells which were dead prior to incubation, typically <10% of the total.

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