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Effects of 5-aminoisoquinolinone, a water-soluble, potent inhibitor of the activity of poly (ADP-ribose) polymerase on the organ injury and dysfunction caused by haemorrhagic shock

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1 Poly (ADP-ribose) synthetase (PARP) is a nuclear enzyme activated by strand breaks in DNA, which are caused *inter alia* by reactive oxygen species (ROS). Here we report on (i) a new synthesis of a water-soluble and potent PARP inhibitor, 5-aminoisoquinolinone (5-AIQ) and (ii) investigate the effects of 5-AIQ on the circulatory failure and the organ injury/dysfunction caused by haemorrhage and resuscitation in the anaesthetized rat.

2 Exposure of human cardiac myoblasts (Girardi cells) to hydrogen peroxide (H_2O_2, 3 mM for 1 h, $n=9$) caused a substantial increase in PARP activity. Pre-treatment of these cells with 5-AIQ (1 μM – 1 mM, 10 min prior to H_2O_2) caused a concentration-dependent inhibition of PARP activity (IC_{50}: $\sim$0.01 mM, $n=6$).

3 Haemorrhage and resuscitation resulted (within 4 h after resuscitation) in a delayed fall in blood pressure (circulatory failure) as well as in rises in the serum levels of (i) urea and creatinine (renal dysfunction), (ii) aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl-transferase (γ-GT) (liver injury and dysfunction), (iii) lipase (pancreatic injury) and (iv) creatine kinase (CK) (neuromuscular injury) ($n=10$).

4 Administration (5 min prior to resuscitation of 5-AIQ) (0.03 mg kg^{-1} i.v., $n=8$, or 0.3 mg kg^{-1} i.v., $n=10$) reduced (in a dose-related fashion) the multiple organ injury and dysfunction, but did not affect the circulatory failure, associated with haemorrhagic shock.

5 Thus, 5-AIQ abolishes the multiple organ injury caused by severe haemorrhage and resuscitation. *British Journal of Pharmacology* (2000) **130,** 843–850

Keywords: 5-Aminoisoquinolinone; haemorrhagic shock; oxygen radicals; poly (ADP-ribose) synthetase; multiple organ failure; trauma

Abbreviations: 3-AB, 3-aminobenzamide; 5-AIQ, 5-aminoisoquinolinone; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATP, adenosine triphosphate; DPQ, 3,4-dihydro-5-[4-(piperidin-1-yl)butoxy]isoquinolin-1(2*H*)-one; γ-GT, γ-glutamyl-transferase; HR, heart rate; MAP, mean arterial blood pressure; NAD, nicotinamide adenine dinucleotide; NO, nitric oxide; PARP, poly (ADP-ribose) polymerase; PARS, poly (ADP-ribose) synthetase; ROS, reactive oxygen species

Introduction

Poly (adenosine 5′-diphosphate ribose) polymerase (PARP) [also known as poly (adenosine 5′-diphosphate ribose) synthetase, PARS; E.C. 2.4.2.30] is ubiquitous, chromatin-bound enzyme (Ikai & Ueda, 1983). Activation of PARP is triggered by single strand breaks in DNA and subsequently catalyses the transfer of ADP-ribose moieties from NAD^+ to various nuclear proteins including histones and PARP (automodification domain) itself (Ueda & Hayaishi, 1985). Continuous or excessive activation of PARP produces extended chains of ADP-ribose on nuclear proteins and results in a substantial depletion of intracellular NAD^+ and subsequently ATP, which may ultimately cause cell death (Berger, 1985; Schraufstatter *et al*., 1986a,b; Hyslop *et al*., 1988; Thies & Autor, 1991). Radicals including superoxide anions, hydrogen peroxide or hydroxyl radicals cause strand breaks in DNA, activation of PARP and depletion of NAD^+ and ATP in cultured cells (Schraufstatter *et al*., 1986a,b; Hyslop *et al*., 1988; Thies & Autor, 1991).

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Severe haemorrhage and resuscitation leads to organ ischaemia (McCord, 1985; Flaherty & Wesfeldt, 1988), the generation of oxygen-derived free radicals upon reperfusion (Zweier *et al*., 1987; Nunes *et al*., 1995), strand breaks in DNA (Carson *et al*., 1986) and ultimately PARP activation. Inhibitors of PARP activity including 3-aminobenzamide (3-AB) reduce the circulatory failure (Szabo *et al*., 1998) and the organ dysfunction/injury (McDonald *et al*., 1999) associated with severe haemorrhagic shock. However, 3-AB is a weak inhibitor of PARP activity that does not readily cross cell membranes (Bowes *et al*., 1998; 1999). Although 1,5-dihydroxyisoquinoline and DPQ, [3,4-dihydro-5-[4-(piperidin-1-yl)butoxy]isoquinolin-1(2*H*)-one] are more potent inhibitors of PARP activity, these agents have to be dissolved in dimethylsulphoxide (DMSO). DMSO itself is a potent scavenger of hydroxyl radicals, and inhibits PARP activity. Thus, there is still a great need for the development of potent, water-soluble inhibitors of PARP activity.

In 1991, Suto and colleagues reported that 5-aminoisoquinolin-1(2*H*)-one (5-AIQ) is a water-soluble inhibitor of PARP activity in a cell-free preparation (enzyme purified

900 fold from calf thymus) (Suto *et al*., 1991). As 5-AIQ is not commercially available, we have optimized the synthetic route (previously described by Wenkert *et al*., 1964) for 5-AIQ. We have then evaluated the effects of 5-AIQ on the circulatory failure and the organ injury and dysfunction (renal dysfunction, liver injury and dysfunction, pancreatic injury) caused by severe haemorrhage and resuscitation in the anaesthetized rat.

Methods

Synthesis of 5-AIQ

Reagents for chemical synthesis were purchased from the Aldrich Chemical Co. (Poole, Dorset, U.K.). NMR spectra were obtained using a Jeol EX400 spectrometer; mass spectra (MS) were obtained using a Fisons VG Autospec Q spectrometer; infra-red (IR) spectra were obtained using a Perkin Elmer 782 spectrometer. Melting points were determined using a Reichert-Jung Thermo Galen instrument and are uncorrected.

5-Nitroisocoumarin Methyl 2-methyl-3-nitrobenzoate (5.0 g, 25.6 mmol) was heated with dimethylformamide dimethylacetal (12 ml) and DMF (30 ml) at 150°C for 16 h. Evaporation, column chromatography (stationary phase: silica gel; mobile phase: hexane/EtOAc 10 : 1) and recrystallization (ethanol) gave 5-nitroisocoumarin (2.43 g, 53%) as white crystals: mp 171 – 172°C (lit. (Somei *et al*., 1981) mp 173 – 174°C); IR (KBr) 1720, 1620 and 1510 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.39 (1 H, dd, $J = 6.0$ Hz, 0.5 Hz, 4-H), 7.44 (1 H, d, $J = 6.0$ Hz, 3-H), 7.68 (1H, t, $J = 8.0$, 7-H), 8.50 (1 H, dd, $J = 8.0$, 1.3 Hz, 6-H), 8.64 (1 H, ddd, $J = 8.0$, 1.3, 0.5 Hz, 8-H).

5-Nitroisoquinolin-1(2H)-one 5-Nitroisocoumarin (1.5 g, 7.8 mmol) was boiled under reflux for 4 h in 2-methoxyethanol (100 ml) saturated with ammonia. Every 30 min, the solution was cooled and ammonia was passed through for 5 min. The solvent was evaporated until 10 ml remained. The solution was stored at 4°C for 16 h. The precipitate was collected, washed (water, then ethanol) to yield 5-nitroisoquinolin-1(2*H*)-one (960 mg, 64%) as orange crystals: mp 247 – 249°C (lit. (Elpern & Hamilton, 1946) mp 250°C); IR (KBr) 1675, 1620 and 1510; ^1H NMR (DMSO-d_6) δ 6.97 (1 H, dd, $J = 7.7$, 0.7 Hz, 4-H), 7.45 (1 H, dd $J = 7.7$, 1.8 Hz (becomes d, $J = 7.7$ Hz on addition of D_2O), 3-H), 7.66 (1 H, t, $J = 7.7$ Hz, 7-H), 8.46 (1 H, dd, $J = 7.7$, 1.5 Hz, 6-H), 8.58 (1 H, ddd, $J = 7.7$, 1.5, 0.7 Hz, 8-H), 11.80 (1 H, br, NH); MS (Cl) 191 (M + H).

5-Aminoisoquinolin-1(2H)-one hydrochloride 5-Nitroisoquinolin-1(2*H*)-one (160 mg, 840 μmol) in ethanol (12 ml) and conc. hydrochloric acid (0.4 ml) was stirred under an atmosphere of hydrogen in the presence of palladium on charcoal (10% 100 mg) for 1 h. The suspension was filtered through Celite®. The Celite® pad and residue were heated in water (200 ml). The resulting hot suspension was filtered through a second Celite® pad. Evaporation of the solvent from the combined filtrates gave 5-aminoisoquinolin-1(2*H*)-one hydrochloride (110 mg, 66%) as white crystals: mp 250 – 260°C (decomp.) ((Wenkert *et al*., 1964) mp 273 – 276°C decomp.); IR (KBr disc) 3410, 3180, 3040-2850 (br), 1685, 1650 cm^{-1}; ^1H NMR (D_2O) δ 6.76 (1 H, d, $J = 7.5$ Hz, 4-H), 7.39 (1 H, d, $J = 7.5$ Hz, 3-H), 7.59 (1 H, t, $J = 8.0$ Hz, 7-H), 7.79 (1 H, d, $J = 8.0$ Hz, 6-H), 8.27 (1 H, d, $J = 8.0$ Hz, 8-H); MS (EI) 161 (M + H), 160 (M).

Cell culture

Human atrial myoblasts (Girardi cells) were obtained from the European Collection of Cell Cultures (Salisbury, Wiltshire, U.K.) and grown to confluence in culture flasks containing Minimum Essential Medium (DMEM) supplemented with L-glutamine (3.5 mM) and 10% foetal calf serum. Cells were passaged every 2 days, removed by treatment with trypsin (0.05%) EDTA (0.02%) and then cultured (37°C, 5% CO_2) in 96-well or 6-well plates (for the measurement of PARP activity only) (Falcon, U.K.) until they reached confluence. Cells were used at the following passage numbers: Girardi cells ($_{P535-545}$). The Girardi cell line derives from a biopsy specimen of the right auricular appendage of an adult human heart.

Experimental design

We have previously reported that exposure of human cardiac myoblasts to hydrogen peroxide causes a concentration-dependent (10 μM – 10 mM) and time-dependent (1 – 6 h) increase in cell injury, which is secondary to an increase in PARP activity in these cells (Bowes *et al*., 1998). To elucidate the effects of 5-AIQ on the cell injury caused by hydrogen peroxide, cells were pre-incubated (10 min, 37°C) with 5-AIQ and then exposed to hydrogen peroxide (3 mM) for 4 h after which time cell injury/death was assessed.

Measurement of cell injury (MTT assay)

Cell viability was determined indirectly by measuring the mitochondrial-dependent reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan (i.e. mitochondrial respiration). Cells in 96-well plates were incubated with MTT (100 μl, 0.2 mg ml^{-1}, dissolved in PBS) for 60 min at 37°C. MTT solution was removed by aspiration and cells were solubilized in 100 μl of dimethylsulphoxide (DMSO). The amount of purple formazan formed was detected and quantified by measuring the absorbance of the solution at 550 nm using an Anthos Labtec microplate reader (Labtec, Uckfield, Sussex, U.K.). Results are expressed as mitochondrial respiration as a percentage of the control cells (i.e. those not exposed to hydrogen peroxide) which was taken as 100%.

Measurement of PARP activity in human cardiomyoblasts

We have previously reported (Bowes *et al*., 1998) that exposure of Girardi cells to hydrogen peroxide causes a concentration-dependent (1 – 10 mM) and time-dependent (10 – 90 min) increase in PARP activity. The study also demonstrated that a maximal increase in PARP activity occurred within 60 min after addition of hydrogen peroxide (3 mM). Hence, the following study was designed to investigate whether 5-AIQ inhibits PARP activity in human cardiac myoblasts. Cells were pre-incubated with media containing the PARP inhibitors 5-AIQ (1 μM – 1 mM). After a 10 min pre-incubation period, the cells were exposed to hydrogen peroxide (3 mM) for 60 min, and then collected to measure PARP activity. PARP activity was measured as the ability of permeabilized cells to transfer the substrate [^3H]-NAD^+ onto nuclear proteins over a set time period as described by Schraufstatter *et al*. (1986a,b). Following the appropriate treatment and duration, the media was aspirated before addition of fresh culture medium (400 μl); the cells were then scraped and transferred to Eppendorff tubes. Following centrifugation (10,000 × *g*, 10 s) and aspiration of media, the

cells were resuspended in reaction buffer (56 mM HEPES buffer containing 28 mM potassium chloride, 28 mM sodium chloride, 2 mM magnesium chloride, 0.02% digitonin, and 125 nmoles NAD^+ spiked with 0.5 μCi ml^{-1} [^{3}H- NAD^+], pH 7.5), vortexed for 5 s and incubated at 37°C for 5 min. The reaction was terminated by addition of 200 μl of 50% trichloroacetic acid (TCA) and the resultant precipitate was pelleted by centrifugation at 10,000 × *g* for 3 min. The protein pellet was washed twice with 50% TCA and was then solubilized in 200 μl 0.3 M NaOH/2% SDS overnight at 37°C in a shaking incubator (Luckham, Basingstoke, Hants., U.K.). The radioactivity incorporated into protein was determined by scintillation counting (Beckman Instruments Ltd, High Wycombe, Bucks., U.K.).

Surgical procedure

This study was carried out on 46 male Wistar rats (Tuck, Rayleigh, Essex, U.K.) weighing 250–320 g receiving a standard diet and water *ad libitum*. All animals were anaesthetized with thiopentone sodium (120 mg kg^{-1} i.p.) and anaesthesia was maintained by supplementary injections (~10 mg kg^{-1} i.v.) of thiopentone sodium as required. In rats, this anaesthetic protocol, which has been approved by the Home Office of the U.K., leads to a long-lasting surgical anaesthesia. The trachea was cannulated to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket. The right femoral artery was catheterized and connected to a pressure transducer (Senso-Nor 840, Senso-Nor, Horten, Norway) for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate (HR) which were displayed on a data acquisition system (MacLab 8e, ADI Instruments, Hastings, U.K.) installed on an Apple Macintosh computer. The right carotid artery was cannulated to facilitate withdrawal of blood (see below). The jugular vein was cannulated for the administration of drugs. The bladder was also cannulated to facilitate urine flow and to prevent the possibility of the development of post-renal failure. Upon completion of the surgical procedure, cardiovascular variables were allowed to stabilise for 15 min. Then, blood was withdrawn from the catheter placed in the carotid artery in order to achieve a fall in MAP to 50 mm Hg within 10 min. Thereafter, MAP was maintained at 50 mm Hg for a total period of 90 min by either withdrawal (during the compensation period) or re-injection of blood. At 90 min after initiation of haemorrhage, the shed blood was re-injected into the animal, along with an equivalent volume of Ringer's Lactate Solution.

Evaluation of the effects of 5-AIQ on the delayed circulatory failure and MODS: experimental design

In the first study aimed at elucidating the effects of 5-AIQ in haemorrhagic shock, all animals were randomized into five groups (see Table 1). Different groups of animals were subjected to 90 min of haemorrhage followed by resuscitation with shed blood and an equivalent volume of Ringer's Lactate Solution for 4 h and treated with either saline (vehicle for 5-AIQ, $n=10$), 5-AIQ (0.03 mg kg^{-1} i.v., $n=8$) or 5-AIQ (0.3 mg kg^{-1} i.v., $n=10$). In addition, we have evaluated the effects of saline (vehicle) or 5-AIQ (0.3 mg kg^{-1} i.v., $n=9$) in rats subjected to the same surgical procedure, but which were not subjected to haemorrhagic shock (sham-operated rats).

Quantification of organ function and injury

Four hours after resuscitation (end of the experiment), 1.5 ml of blood was collected into a serum gel S/1.3 tube (Sarstedt, Germany) from the catheter placed in the right carotid artery. The blood sample was centrifuged (1610 × *g* for 3 min at room temperature) to separate plasma. All plasma samples were analysed within 24 h by a contract laboratory for veterinary clinical chemistry (Vetlab Services, Sussex, U.K.). The following marker enzymes were measured in the plasma as biochemical indicators of multiple organ injury/dysfunction: (1) Renal dysfunction was assessed by measuring the rises in plasma levels of creatinine (an indicator of impaired renal function) and urea (an indicator of impaired excretory function of the kidney and/or increased catabolism) (see Thiemermann *et al*., 1995); (2) Liver injury was assessed by measuring the rise in plasma levels of alanine aminotransferase (ALT, an indicator of hepatic parenchymal injury), aspartate aminotransferase (AST, a non-specific marker for hepatic injury), and γ-glutamyl-transferase (γ-GT, indicator of liver dysfunction and injury) (Baue, 1993); (3) In addition, we have evaluated the rises in the serum levels of lipase, a specific indicator for the development of pancreatic injury and (4) Finally, we determined the increase in the serum levels of creatine kinase, an indicator for the development of muscle (skeletal or cardiac) or brain injury.

In order to ensure that 5-AIQ does not interfere with the determination of any of the above parameters of organ injury, we have carried out the following experiment. Serum (250 μl) was obtained from seven rats (four subjected to haemorrhagic shock and three subjected to the surgical procedure) and then spiked with either 250 μl of saline or with 250 μl containing 7.2 μg ml^{-1} of 5-AIQ. Given an estimated blood volume of ~25 ml of blood per rat, we have estimated the maximal concentration of 5-AIQ in the blood to be 3.6 μg ml^{-1} (or 18.3 μM). The above assay has taken this estimation into account and, hence, the final concentration of 5-AIQ in the sample (comprising of 250 μl of serum plus 250 μl of saline containing 5-AIQ) was 3.6 μg ml^{-1} (or 18.3 μM). We have then subjected this sample to analysis and determined the following parameters: urea, creatinine, AST, ALT, CK and lipase.

Table 1 Experimental design

Group	*Protocol*	*Treatment*	*Dose*	n
1	Sham	Vehicle (saline)		9
2	Sham	5-aminoisoquinolinone	0.3 mg kg^{-1}	9
3	HS	Vehicle (saline)		10
4	HS	5-aminoisoquinolinone	0.03 mg kg^{-1}	8
5	HS	5-aminoisoquinolinone	0.3 mg kg^{-1}	10

Animals were subjected to the surgical procedure alone (sham) or to haemorrhagic shock (HS).

Materials

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company Ltd. (Poole, Dorset, U.K.). Minimum Essential medium (DMEM) was from Life Technology Ltd. (Paisley, Lanarkshire, U.K.). Thiopentone sodium (Intraval Sodium®) was obtained from Rhône Mérieux Ltd. (Harlow, Essex, U.K.). All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, U.K.).

Statistical evaluation

All data are presented as mean ± s.e.mean of *n* observations, where *n* represents the number of animals or blood samples

studied. The IC_{50} values were calculated using a Graph Pad Prism Statistical Package (Version 3.0). For repeated measurements (haemodynamics) a 2-factorial analysis of variance (ANOVA) was performed. Data without repeated measurements (multiple organ injury/failure) was analysed by 1-factorial ANOVA, followed by a Dunnett's test for multiple comparisons using a Graph Pad Prism Statistical Package (Version 3.0). A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Synthesis of 5-AIQ

In the Methods section of this study, we describe a new, reliable and efficient synthesis of the target compound, 5-aminoisoquinolin-1(2*H*)-one (5-AIQ) (Figure 1). Wenkert *et al*. (1964) reported synthesis of 5-AIQ by reduction of 5-nitroisoquinolinone, but the preparation of this latter compound (N-oxidation of 5-nitroquinoline and rearrangement of the 5-nitroisoquinoline-N-oxide with hot acetic anhydride) was low yielding and unreliable in our hands (M.D. Threadgill and C.Y. Watson, data not shown). 5-Nitroisoquinolinone is also not amenable to synthesis by thermolytic Curtius rearrangement and cyclization, a general route to 5-substituted isoquinolinones (Watson *et al*., 1998; Eloy & Deryckere, 1969), as the nitro group is powerfully electron-withdrawing. As other isoquinolinones can be synthesized by treatment of the corresponding isocoumarins by treatment with ammonia at high temperature (Watson *et al*., 1998; Parveen *et al*., 1999), we rationalized that an improved preparation of 5-nitroisoquinolinone may be achieved by this method. Condensation of methyl 2-methyl-3-nitrobenzoate (Askam & Keeks, 1969) with dimethylformamide dimethylacetal at high temperature gave the enamine methyl 2-(2-*Z*-dimethylaminoethenyl)-3-nitrobenzoate. Immediate passage through of silica gel hydrolyzed of the enamine and cyclized the intermediate enol to give 5-nitroisocoumarin in good yield. Treatment with boiling ammonia-saturated 2-methoxyethanol afforded 5-nitroisoquinolinone, which was converted to 5-AIQ by hydrogenolysis. Thus, our improved synthesis of 5-AIQ gives the target compound in 18% overall yield without recourse to difficult reagents or elevated pressures.

Effects of 5-AIQ on the impairment in mitochondrial respiration caused by hydrogen peroxide in human cardiac myoblasts

Exposure of human cardiac myoblasts to hydrogen peroxide (3 mM for 4 h) caused a substantial (~80%) reduction in mitochondrial respiration (Figure 2). Pre-treatment of these cells with 5-AIQ caused a concentration-dependent attenuation (IC_{50}: ~0.014 mM) of the impairment in mitochondrial respiration caused by hydrogen peroxide (Figure 2).

Effects of 5-AIQ on the increase in PARP activity caused by hydrogen peroxide in human cardiac myoblasts

Exposure of human cardiac myoblasts to hydrogen peroxide (3 mM for 1 h) caused a 5 fold increase in PARP activity (Figure 3). This increase in PARP activity was attenuated by pre-treatment of the cells with 5-AIQ in a concentration-dependent fashion (IC_{50}: ~0.012 mM).

Effects of 5-AIQ on the delayed vascular decompensation (circulatory failure) caused haemorrhage

In rats subjected to haemorrhage, resuscitation with shed blood led to an immediate increase in blood pressure from

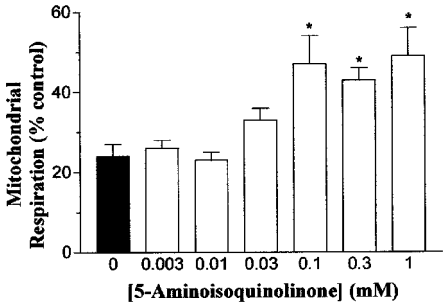

Figure 2 The effect of 5-AIQ (0.01–10 mM, $n=4$-6) on the impairment in mitochondrial respiration caused by hydrogen peroxide (H_2O_2, 3 mM, $n=6$) in human cardiac myoblasts. 5-AIQ causes a concentration-dependent attenuation of the impairment in mitochondrial respiration caused by H_2O_2. Data are expressed as mean ± s.e.mean of n observations $^*P<0.05$ when compared with H_2O_2-control (solid column).

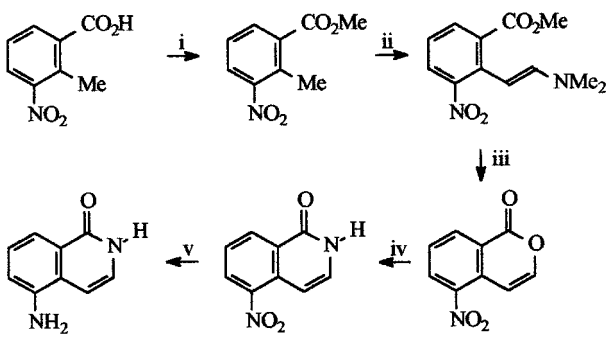

Figure 1 Synthetic route to 5-aminoisoquinolin-1(2*H*)-one (5-AIQ). *Reagents*: i, methanol, thionyl chloride, heat; ii, dimethylformamide dimethylacetal, DMF, heat; iii, silica gel; iv, ammonia, 2-methoxyethanol, heat; v, hydrogen, 10% palladium on charcoal, hydrochloric acid.

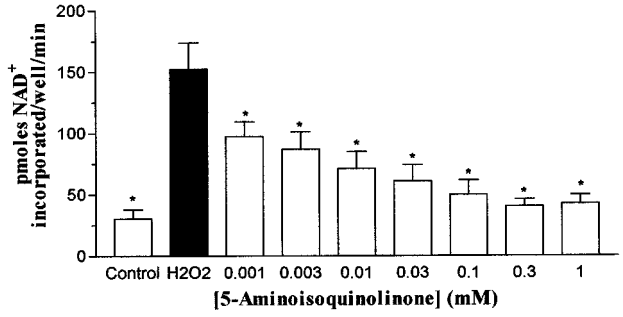

Figure 3 The effect of 5-AIQ (0.01 – 10 mM, $n=6$) on the increase in PARP activity caused by hydrogen peroxide (H_2O_2, 3 mM, $n=9$) in human cardiac myoblasts. 5-AIQ causes a concentration-dependent attenuation of the increase in PARP activity caused by H_2O_2. Data are expressed as mean ± s.e.mean of n observations $^*P<0.05$ when compared with H_2O_2-control (solid column).

$\sim$50 to 108$\pm$5 mm Hg. Thereafter, there was a progressive decline in MAP to approximately 75 mm Hg at the end of the experiment (Figure 4). The PARP inhibitor 5-AIQ did not significantly attenuate the delayed fall in MAP associated with haemorrhage at any of the time points measured (Figure 4, $P>0.05$). In sham-operated rats, neither administration of saline nor administration of the PARP inhibitors had any effect on MAP (Figure 1) or HR (Table 2). Haemorrhagic shock did not cause a significant alteration in heart rate (Table 2, $P>0.05$).

Effects of 5-AIQ on the multiple organ dysfunction syndrome caused by haemorrhage in the rat

When compared with sham-operated rats, haemorrhage/resuscitation resulted in significant rises in the plasma levels of urea, creatinine (renal dysfunction, Figure 5), AST, ALT, γ-GT (liver injury, Figure 6), lipase (pancreatic injury, Figure 7) or CK (Figure 8). Treatment of rats subjected to haemorrhage and resuscitation with 5-AIQ (0.03 or 0.3 mg kg^{-1} i.v.) attenuated the organ injury and dysfunction caused by haemorrhage and resuscitation (Figures 5–8). In sham-operated rats, neither administration of saline nor administration of the PARP inhibitor had any effect on the biochemical indicators of organ injury/dysfunction (Figures 5–8). In a separate set of experiments, plasma samples obtained from rats subjected to haemorrhagic shock or to sham-operation were spiked with 5-AIQ in order to evaluate whether 5-AIQ affects the determination of any of the above parameters of organ injury. The PARP inhibitor 5-AIQ, however, had no significant effect on the determination of creatinine, urea, AST, ALT, γ-GT, lipase or CK (Table 3).

Discussion

In this study, we describe a new, reliable and efficient synthesis of the target compound, 5-amino-isoquinolin-1(2*H*)-one (5-AIQ) (Figure 1). This method leads to a higher yield of 5-AIQ than the one reported by Wenkert *et al.* (1964). Suto and colleagues have reported that 5-AIQ has an IC_{50} of 240 nM when evaluated *in vitro* in a cell-free system (isolated PARP enzyme isolated from calf thymus) for inhibitory activity against PARP, which is broadly comparable with other potent 5-substituted isoquinolinones (Watson *et al.*, 1998; Suto *et al.*, 1991). As the compound is a mimic of the nicotinamide moiety of the substrate NAD^+, it is conceivable that it may also inhibit other ADP-ribosyl transferases. Thus, we examined its activity against the mono-ADP-ribosylating activity of diphtheria toxin and found that it had an IC_{50} of approximately 250 μM, indicating *ca.* 1000 fold selectivity for PARP inhibition (S. Oldfield and M.D. Threadgill, unpub-

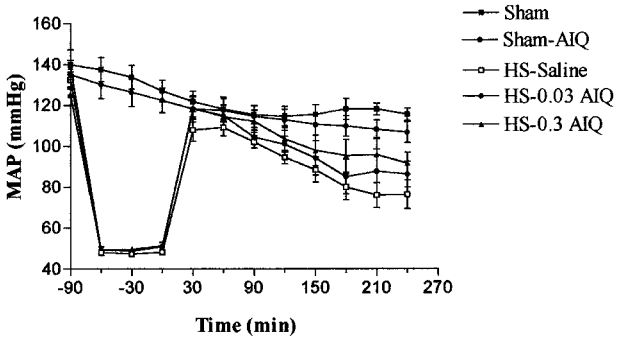

Figure 4 Alterations in the mean arterial pressure (MAP). Animals were subjected to the surgical procedure alone and treated with saline ($n=9$) or to haemorrhagic shock and treated with vehicle ($n=10$), 5-AIQ (0.03 mg kg^{-1} i.v., $n=8$), or 5-AIQ (0.3 mg kg^{-1} i.v., $n=10$). *$P<0.05$ when compared to HS-saline.

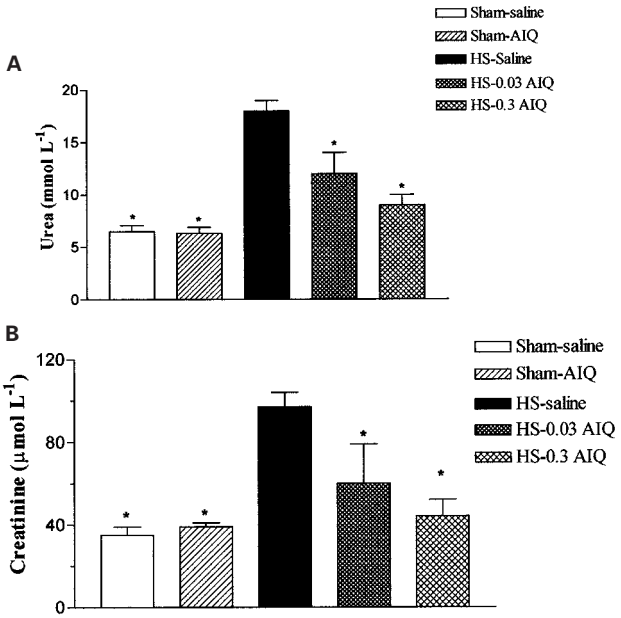

Figure 5 Alterations in the serum levels of urea (A) and creatinine (B). Animals were subjected to the surgical procedure alone and treated with saline ($n=9$) or to haemorrhagic shock and treated with vehicle ($n=10$), 5-AIQ (0.03 mg kg^{-1} i.v., $n=8$), or 5-AIQ (0.3 mg kg^{-1} i.v., $n=10$). *$P<0.05$ when compared to HS-saline.

Table 2 Alterations in heart rate (HR)

			Resuscitation (min)							
Group	n	*Baseline*	*30*	*60*	*90*	*120*	*150*	*180*	*210*	*240*
HS + saline	10	415$\pm$14	401$\pm$13	417$\pm$9	412$\pm$6	432$\pm$11	412$\pm$13	416$\pm$17	406$\pm$21	426$\pm$15
HS + AIQ (0.03 mg kg^{-1})	8	403$\pm$12	391$\pm$15	398$\pm$19	404$\pm$12	393$\pm$9	371$\pm$22	384$\pm$21	386$\pm$11	399$\pm$24
HS + AIQ (0.3 mg kg^{-1})	10	364$\pm$7	371$\pm$6	393$\pm$9	399$\pm$11	384$\pm$13	388$\pm$12	395$\pm$9	396$\pm$11	375$\pm$14
Sham + saline	9	387$\pm$14	360$\pm$16	365$\pm$14	264$\pm$19	373$\pm$15	379$\pm$16	374$\pm$11	366$\pm$12	365$\pm$14
Sham + AIQ (0.3 mg kg^{-1})	9	399$\pm$16	378$\pm$12	367$\pm$9	368$\pm$13	383$\pm$12	391$\pm$14	392$\pm$17	382$\pm$13	382$\pm$13

Animals were subjected to the surgical procedure alone (sham) and treated with saline (vehicle, $n=9$), 5-aminoisoquinolinone (5-AIQ, 0.3 mg kg^{-1} i.v., $n=9$). In separate experiments, animals were subjected to haemorrhagic shock (HS) and treated with saline (vehicle, $n=10$), 5-AIQ (0.03 mg kg^{-1} i.v., $n=8$) 5-AIQ (0.3 mg kg^{-1} i.v., $n=10$).

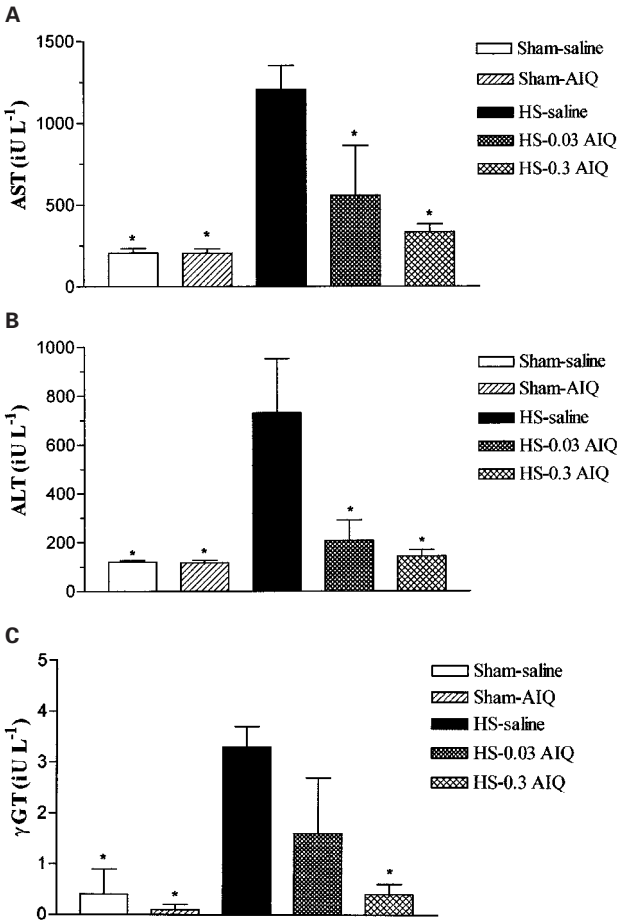

Figure 6 Alterations in the serum levels of asparate aminotransferase (AST, (A)), alanine aminotransferase (ALT, (B)), γ-glutamyl transferase (γ-GT, (C)). Animals were subjected to the surgical procedure alone and treated with saline ($n=9$) or to haemorrhagic shock and treated with vehicle ($n=10$), 5-AIQ (0.03 mg kg^{-1} i.v., $n=8$), or 5-AIQ (0.3 mg kg^{-1} i.v., $n=10$). *$P<0.05$ when compared to HS-saline.

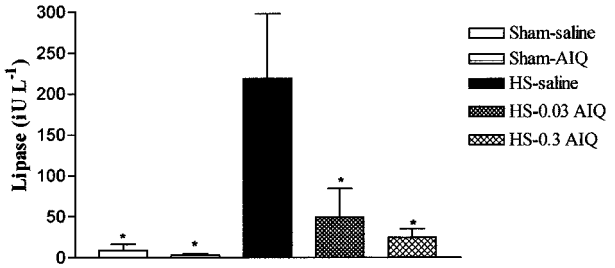

Figure 7 Alterations in the serum levels of lipase. Animals were subjected to the surgical procedure alone and treated with saline ($n=9$) or to haemorrhagic shock and treated with vehicle ($n=10$), 5-AIQ (0.03 mg kg^{-1} i.v., $n=8$), or 5-AIQ (0.3 mg kg^{-1} i.v., $n=10$). *$P<0.05$ when compared to HS-saline.

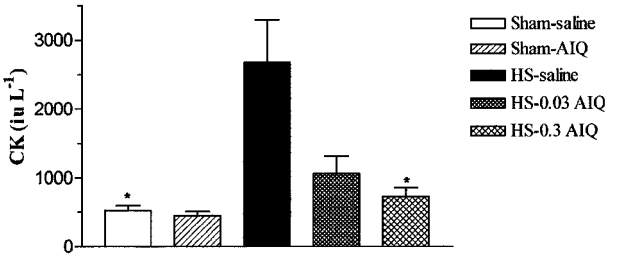

Figure 8 Alterations in the serum levels of creatine kinase (CK). Animals were subjected to the surgical procedure alone and treated with saline ($n=9$) or to haemorrhagic shock and treated with vehicle ($n=10$), 5-AIQ (0.03 mg kg^{-1} i.v., $n=8$), or 5-AIQ (0.3 mg kg^{-1} i.v., $n=10$). *$P<0.05$ when compared to HS-saline.

Table 3 Effects of 5-AIQ on the determination of biochemical parameters

	Spiking solution	
Biochemical parameter	*Saline* ($n=7$)	*AIQ* ($n=7$)
Urea (mmol l^{-1})	6.0±1	6.0±1
Creatinine (μmol l^{-1})	16±1	16±2
AST (iu l^{-1})	112±21	118±23
ALT (iu l^{-1})	64±8	65±9
γ-GT (iu l^{-1})	0±0	0±0
Lipase (iu l^{-1})	19±3	10±4
CK (iu l^{-1})	190±38	246±58

Plasma samples (250 μl) were spiked with 250 μl of saline or 250 μl of saline containing 5-AIQ (36.6 μM). Samples were then subjected to biochemical analysis.

lished results). Armed with this encouraging potency and enzyme selectivity *in vitro* and with the great advantage of very good water-solubility of 5-AIQ in comparison with other isoquinolinones, we proceeded to investigate the effects of this compound on PARP activity in human cardiac myoblasts. Exposure of human cardiac myoblasts to hydrogen peroxide (3 mM for 1 h) resulted in a 5 fold increase in PARP activity. This increase in PARP activity was attenuated by pre-treatment of the cells with 5-AIQ in a concentration-dependent fashion (IC_{50}: ~0.012 mM). The inhibition by 5-AIQ of the increase in PARP activity in human cardiac myoblasts was associated with a reduction in the impairment in mitochondrial respiration caused by hydrogen peroxide in these cells. These findings demonstrate that 5-AIQ is a water soluble and potent inhibitor of PARP activity. When compared to 3-AB (EC_{50}: ~0.51 mM), 1,5-dihydroxyisoquinoline (5-hydroxyisoquinolin-1(2*H*)-one) (EC_{50}: ~0.02 mM), or nicotinamide (EC_{50}: ~0.78 mM) (Bowes *et al*., 1998), 5-AIQ is the most potent inhibitor of PARP activity which we have tested in this human cell line. In a separate study, we have then evaluated the effects of 5-AIQ on the circulatory and multiple organ failure associated with haemorrhagic shock in the rat. Haemorrhage for 90 min followed by resuscitation with shed blood (for 4 h) resulted in (i) acute renal dysfunction, (ii) hepatocellular injury, (iii) pancreatic injury and (iv) neuromuscular injury. We have previously confirmed (by using light microscopy) that the model of haemorrhagic shock used here results in a substantial degree of tissue injury to the lung, kidney, intestine and liver (Mota-Filipe *et al*., 1999). We report here that the administration (at 5 min prior to resuscitation) of 5-AIQ (0.3 mg kg^{-1} i.v.) abolishes (i) the renal dysfunction, (ii) the liver injury and dysfunction, (iii) the pancreatic injury and (iv) the muscle and/or brain injury caused by haemorrhage and resuscitation. Even the lower dose of 5-AIQ used (0.03 mg kg^{-1} i.v.) caused a significant reduction in several of the parameters, e.g. pancreatic injury. It should be noted that the doses of 5-AIQ, which we have used here, are substantially lower than those of 3-AB (10 mg kg^{-1} i.v.), or 1,5-dihydroxyisoquinoline (1–3 mg kg^{-1} i.v.) used in this model of haemorrhagic shock (McDonald *et al*., 1999), or in

other *in vivo* studies aimed at elucidating the role of PARP, in conditions associated with ischaemia-reperfusion and/or inflammation.

In principle, severe haemorrhage followed by resuscitation leads to ischaemia and reperfusion (injury) of target organs including the heart, liver, brain and kidney (Flaherty & Wesfeldt, 1988). There is good evidence that less potent inhibitors of PARP activity (including 3-AB: 10 mg kg^{-1}; nicotinamide: 10 mg kg^{-1} and 1,5-dihydroxyisoquinoline: 3 mg kg^{-1}) reduce by $\sim$30–50% the degree of tissue injury associated with regional myocardial ischaemia and reperfusion of the heart (Thiemermann *et al*., 1997; Zingarelli *et al*., 1997; 1998; Bowes *et al*., 1999), the brain (Eliasson *et al*., 1997), the gut (Cuzzocrea *et al*., 1997) and the kidney (Chatterjee *et al*., 1999). Interestingly, a much larger reduction in cerebral infarct size ($\sim$80%) can be detected in mice in which the gene for PARP has been disrupted by gene-targeting (PARP knock out or $-/-$ mice) (Eliasson *et al*., 1997). Thus, it is possible that a much larger therapeutic benefit in conditions associated with ischaemia-reperfusion, can be obtained with more potent, water-soluble inhibitors of PARP activity. The effects of 5-AIQ on the infarct size arising from ischaemia-reperfusion of the brain, heart and kidney, therefore, warrant investigation.

The PARP inhibitor 3-AB (15 mg kg^{-1}) has been reported to attenuate the delayed circulatory failure (e.g. fall in blood pressure, cardiac output and stroke volume) associated with severe haemorrhage in the pig. Thus, it has been suggested (Szabo *et al*., 1998) that the beneficial effects of 3-AB in haemorrhagic shock are due to an improved cardiac performance. We have not measured the effects of any of the PARP inhibitor 5-AIQ on cardiac performance. In our study, 5-AIQ had no significant effect on blood pressure in rats subjected to haemorrhagic shock.

In conclusion, this study reports on the synthesis of 5-AIQ and demonstrates that this agent is a very potent inhibitor of PARP activity in human cardiac myoblasts. We have also discovered that 5-AIQ (0.3 mg kg^{-1}) abolishes the multiple organ injury and dysfunction caused by severe haemorrhage and resuscitation. Our results are consistent with the hypothesis (McDonald *et al*., 1999) that severe haemorrhage and resuscitation leads to organ ischaemia (McCord, 1985; Flaherty & Wesfeldt, 1988), the generation of oxygen- or nitrogen-derived free radicals upon reperfusion (Zweier *et al*., 1987; Nunes *et al*., 1995), strand breaks in DNA (Carson *et al*., 1986) and ultimately PARP activation. The resultant excessive activation of PARP contributes to the organ injury and dysfunction associated with severe haemorrhage and resuscitation.

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