British Journal of Pharmacology (2004) 142, 953–960 
www.nature.com/bjp

# Inhibition of c-Jun N-terminal kinase decreases cardiomyocyte apoptosis and infarct size after myocardial ischemia and reperfusion in anaesthetized rats

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**1** Myocardial ischemia/reperfusion is associated with inflammation, apoptosis and necrosis. During this process, c-jun N-terminal kinase is activated in cardiac myocytes resulting in apoptosis.

**2** This study investigates the effects of AS601245, a nonpeptide ATP competitive JNK inhibitor, on infarct size caused by myocardial ischemia/reperfusion in anaesthetized rats. The left descending coronary artery of anaesthetized rats was occluded for 30 min and then reperfused for 3 h. AS601245 was administered 5 min before the end of the ischemia period as an i.v. bolus (1.5, 4.5 or 15 mg kg$^{-1}$ i.v.) followed by continuous i.v. infusion (18, 55 and 183 $\mu$g kg$^{-1}$ min$^{-1}$, respectively) during reperfusion. Controls received saline only. 3-Aminobenzamide, a poly(ADP-ribose) polymerase inhibitor, was used as reference compound at 10 mg kg$^{-1}$ i.v. bolus plus 0.17 mg kg$^{-1}$ min$^{-1}$ continuous infusion.

**3** AS601245 significantly reduced infarct size at 4.5 mg kg$^{-1}$ (−44%; $P<0.001$) and 15 mg kg$^{-1}$ i.v. (−40.3%; $P<0.001$) similarly to 3-aminobenzamide (−44.2%; $P<0.001$). This protective effect was obtained without affecting hemodinamics or reducing ST-segment displacement.

**4** The beneficial effects on infarct size correlated well with the reduction of c-jun phosphorylation (−85%; $P<0.001$ *versus* control) and of TUNEL-positive cells (−82.1%; $P<0.001$) in post-ischemic cardiomyocytes. No change in the phosphorylation state of p38 MAPK and ERK in post-ischemic heart was observed in the presence of AS601245 in comparison to the vehicle-treated group.

**5** These results demonstrate that blocking the JNK pathway may represent a novel therapeutic approach for treating myocardial ischemia/reperfusion-induced cardiomyocyte death.
*British Journal of Pharmacology* (2004) **142,** 953–960. doi:10.1038/sj.bjp.0705873

**Keywords:** Myocardial ischemia/reperfusion, JNK, apoptosis, JNK inhibitor

**Abbreviations:** AAR, area at risk; 3-AB, 3-aminobenzamide; AS601245, (1,3-benzothiazol-2-yl (2-{[2-(3-pyridinyl) ethyl] amino}-4 pyrimidinyl) acetonitrile); ATP, adenosine triphosphate; HR, heart rate; IS, infarct size; JNK, c-jun N-terminal kinase; LAD, left anterior descending coronary artery; MAP, mean arterial pressure; NBT, *p*-nitro-blue tetrazolium; p38 MAPK, p38 mitogen-activated protein kinase; p42 MAPK, p42 mitogen-activated protein kinase; p44 MAPK, p44 mitogen-activated protein kinase; PRI, pressure rate index; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin *in situ* nick-end labelling

## Introduction

Apoptosis is an active gene-directed cell death process which plays a key role in myocardial reperfusion injury (Gottlieb *et al*., 1994; Olivetti *et al*., 1997; Schumann *et al*., 1997; Barling *et al*., 1998; Aoki *et al*., 2002). Cardiac myocyte cell death triggered by ischemia/reperfusion can occur by both apoptosis and necrosis. While cell death after prolonged periods of ischemia is ascribed to necrosis, apoptosis occurs in cells and tissues exposed to reoxygenation after ischemia. The intracellular signaling pathways that mediate stress responses of cardiomyocytes are not fully delineated. However, it has recently been demonstrated that myocardial ischemia/reperfusion activates the two 'stress-responsive' mitogen-activated protein kinase (MAPK) subfamilies, namely, c-Jun N-terminal kinases (JNK) and p38 mitogen-activated protein kinases (p38–MAPKs), resulting in apoptosis (Yin *et al*., 1997; Sugden & Clerk, 1998). While p38-MAPK is activated by ischemia and this activation state is maintained during reperfusion (Bogoyevitch *et al*., 1996; Hreniuk *et al*., 2001), activation of JNK occurs only during reperfusion (Knight & Buxton, 1996; Laderoute & Webster, 1997; Clerk *et al*., 1998). Therefore, inhibition of apoptosis could be a therapeutic approach for reducing myocardial damage after oxidative stress.

The benzothiazole derivative AS601245 (1,3-benzothiazol-2-yl(2-{[2-(3-pyridinyl)ethyl] amino}-4-pyrimidinyl) acetonitrile) is a new, potent adenosine triphosphate (ATP) competitive JNK inhibitor and a nonspecific inhibitor of the three human JNK isoforms namely JNK1, JNK2 and JNK3, which

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Advance online publication: 21 June 2004

corresponds to SAPK$\gamma$, SAPK$\alpha$ and SAPK$\beta$ in rats (Gupta *et al*., 1996), with $IC_{50}$'s of 150, 220 and 70 nM, respectively. AS601245 is endowed with a selectivity profile against a panel of serine/threonine and tyrosine kinases (Table 1): eight-fold selectivity against c-Raf, c-src and CDK2/CycA, 30-fold against p38a and 60-fold against ERK1, MEK1, PKC, AKT, p56Lck and EGF (Carboni *et al*., 2004). In cells, AS601245 at 3–10 $\mu$M, inhibited by 30–40% respectively the NGF and serum deprivation-induced neuronal cell death in human superior cerebellar ganglia cells and in human teratocarcinoma cells (differentiated to neurons), (Pascale Gaillard, personal communication). In addition, AS601245 has shown beneficial effects in models of global and focal brain ischemia (Carboni *et al*., 2004). Therefore, this study aimed to determine the potential effects of AS601245 in reducing cardiomyocyte apoptosis and in limiting infarct size (IS) after myocardial ischemia/reperfusion in anaesthetized rats (Baller *et al*., 1981; Zacharowski *et al*., 1999) and, most importantly, to explore its molecular mechanism of action.

## Methods

### *Left anterior descending (LAD) coronary occlusion and reperfusion*

Male Sprague–Dawley rats (300–400 g; Charles River, Calco, Italy) were anaesthetized with sodium pentobarbital (45 mg kg$^{-1}$ i.p. plus 0.05–0.2 mg min i.v.) and mechanically ventilated (1 ml 100 g$^{-1}$ B.W; 60 strokes min$^{-1}$). LAD occlusion was induced according to Zacharowski *et al*. (1999). Heart rate (HR), mean arterial blood pressure (MAP) and lead II ECG were continuously recorded by PO-NE-MAH (PO-NE-MAH Inc., Simsbury, CT, U.S.A.) data acquisition/analysis system. The pressure rate index (PRI), as indicator of myocardial oxygen consumption, was calculated as the product of MAP and HR (Baller *et al*., 1981). AS601245 (Chemistry Department, Serono Pharmaceutical Research Institute, Geneva, Switzerland) was administered 5 min before the end of ischemia as an i.v. bolus (1.5, 4.5 and 15 mg kg$^{-1}$ i.v.; $n=8$ group$^{-1}$) followed by continuous i.v. infusion (18, 55 and 183 $\mu$g kg$^{-1}$ min$^{-1}$) throughout reperfusion. These dose regimens were proven to provide targeted plasma levels of 1, 3 and 10 $\mu$g ml$^{-1}$ (data not shown). The control group received saline only, while another group received 3-aminobenzamide (3-AB), a poly(ADP-ribose)polymerase inhibitor known to exert a cardioprotective action in rat models of myocardial ischemia/reperfusion (Liaudet *et al*., 2001; Chiarugi, 2002) at 10 mg kg$^{-1}$ as an i.v. bolus plus 0.17 mg kg$^{-1}$ min$^{-1}$ by continuous infusion ($n=8$ group$^{-1}$). Three additional groups of nine rats each (one sham-operated, and two groups subjected to 30-min ischemia followed by 3-h reperfusion, receiving saline or AS601245 at 4.5 mg kg$^{-1}$ followed by continuous i.v. infusion of 55 $\mu$g kg$^{-1}$ min$^{-1}$) were included to perform DNA laddering and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin *in situ* nick-end labelling) assays, immunohistochemistry and Western blot analysis on post-ischemic hearts. For clarity from now inwards, dosages are indicated as 1.5, 4.5 and 15 mg kg$^{-1}$ i.v. for AS601245 and 10 mg kg$^{-1}$ i.v. for 3-AB, corresponding to a total dose of 4.7, 14.4 and 47.9 mg kg$^{-1}$ for AS601245 and 40.8 mg kg$^{-1}$ for 3-AB.

All *in vivo* studies were performed according to the European Council Directive 86/609/EEC and the Italian Ministry guidelines for the care and use of experimental animals (decree # 116/92). This experimental protocol was authorized by the Italian Ministry of Health.

**Table 1** AS601245 kinase selectivity profile

| *Kinases* | $IC_{50}$ (μM) | *Kinases* | $IC_{50}$ (μM) |
|---|---|---|---|
| JNK3 | 0.07 | MKK7b | >10 |
| JNK2 | 0.22 | MKK4 | >10 |
| JNK1 | 0.15 | MAPKAP-K2 | >10 |
| | | ERK1 | >10 |
| c-SRC | 1.1 | MEK1 | >10 |
| c-Raf | 1.2 | PI3Kg | >10 |
| CDK2/CycA | 1.4 | PDK1 | >10 |
| P38a | 4.8 | AKT | >10 |
| | | p70S6K | >10 |
| MKK6 | 5–10 | P56Lck | >10 |
| RSK-2 | 5–10 | CHK1 | >10 |
| ROCK-II | 5–10 | EGF | >10 |
| Blk | 5–10 | IKKb | >10 |
| MSK1 | 5–10 | PKC | >10 |
| SGK | 5–10 | PRAK | >10 |

Protein kinases were assayed for their ability to phosphorylate the appropriate peptide/protein substrates. Assays were performed using 10 $\mu$M ATP. AS601245 was challenged at 10 $\mu$M in triplicate. When $IC_{50}$ value were determined, AS601245 was incubated at 5–7 increasing concentrations in triplicate. For other method details, refers to Carboni *et al*. (2004).

### *IS determination*

Following 3 h of reperfusion, the LAD was ligated again, and 3 ml kg$^{-1}$ of 1% Evans blue were administered i.v. to stain the area at risk (AAR). The heart was then removed and transversally divided into 4–5 slices of 1–2 mm width. The Evans blue solution stained the perfused myocardium leaving the occluded vascular bed uncolored. All the coloured non-ischemic tissue and the non-colored AAR were weighed to calculate the percentage of the AAR with respect to the whole left ventricle. To distinguish between viable ischemic and infarcted tissue, the AAR was cut into small pieces and incubated with *p*-nitro-blue tetrazolium (NBT, 0.5 mg ml$^{-1}$, 20 min at 37°C). In the presence of intact dehydrogenase enzyme systems (normal myocardium), NBT forms dark blue formazan while areas of necrosis lacking dehydrogenase activity do not stain. IS was then calculated and expressed as percentage of AAR (Zacharowski *et al*., 1999).

### *DNA fragmentation (DNA ladder)*

Hearts were frozen and stored at –70°C, then minced while being thawed in lysis buffer (50 mmol l$^{-1}$ Tris-HCl, pH 8.0; 20 mmol l$^{-1}$ EDTA and 1% SDS) on ice. Proteinase K (100 $\mu$g ml$^{-1}$) was then added. After incubation at 55°C with shaking for 18 h, DNA was extracted three times with phenol/chloroform, precipitated in ethanol, treated with DNAse-free RNAse, re-extracted, and precipitated again. DNA concentration was then determined. Next, 5 $\mu$g of DNA were subjected to electrophoresis on 1.2% ethidium bromide-stained agarose gels and oligonucleosomal DNA fragmentation was visualized (Yue *et al*, 1997).

### TUNEL

Hearts were washed by perfusion with ice-cold PBS for 1 min followed by a fixation step with ice-cold 4% paraformaldehyde in phosphate buffer (peristaltic pump flow: 50 ml $min^{-1}$ for 10 min). After 24 h in fixation solution, hearts were cryosectioned at a thickness of 10 $\mu$m and serial sections were stained with TUNEL reagents (*In Situ* Cell Death Detection, POD; Roche, Mannheim, Germany), according to the manufacturer's instructions. Cell type was identified by hematoxylin staining (Vector Laboratories, Burlingame, CA, U.S.A.). Nuclei were counted in 8–10 microscopic fields for each heart. The mean number of nuclei per $mm^2$ was multiplied by the section area to calculate the total nuclei present. The number of TUNEL-positive cardiomyocytes in 8–10 fields was divided by the total cardiomyocyte number to determine the ratio of TUNEL-positive cells.

### Immunohistochemistry

Paraformaldeyde-fixed hearts were cryosectioned at a thickness of 10 $\mu$m and prepared for immunoperoxidase staining using a Vectastain ABC kit (Vector Laboratories). Briefly, endogenous peroxidase was quenched with 0.3% $H_2O_2$ in methanol for 30 min. Nonspecific immunoglobulin binding sites were blocked with normal goat serum for 1 h, then sections were incubated with the primary antibody anti-phospho-c-jun (Ser 73) and anti-c-jun (Cell Signaling Technology, Beverly, MA, U.S.A.) for 1 h at room temperature. The sections were then incubated for 30 min with a biotinylated secondary antibody solution followed by 30 min incubation with ABC reagent (Vectastain Elite ABC kit). Immunoglobulin complexes were visualized on incubation with 3,3′-diaminobenzidine (Sigma, St Louis, MO, U.S.A.), then washed, counterstained with hematoxylin, cleared, mounted and examined by light microscopy. A total of 10 fields were observed for each heart. As negative control for the immunohistochemical staining, tissue sections were treated with normal serum instead of primary antibodies. Conventional histological observation was performed using hematoxylin and eosin staining.

### Western blotting

Heart tissues were lysed with ice-cold lysis buffer (62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w $v^{-1}$ SDS, 10% glycerol, 50 mM DTT, 0.01% w $v^{-1}$ bromophenol blue or phenol red). After sonication, lysates were centrifuged, protein concentration determined and 20 $\mu$g of proteins separated by electrophoresis on 10% SDS–PAGE and transferred onto a polyvinylidene difluoride-plus membrane. After blocking with 5% milk, the immunoblots were probed with a 1 : 100 dilution anti-phospho-c-jun (Ser 73), anti-phospho-SEK1/MKK4 (Thr 261), anti-p46/p54-SAPK/JNK (Thr 183/Tyr 185), anti-phospho-p38 MAPK (Thr 180/Thr 182), anti-phospho-p44/42 MAPK (Thr202/Thr204) antibodies (Cell Signaling Technology) overnight at 4°C, followed by 1 h incubation at room temperature with the corresponding secondary antibodies. The blots were visualized with ECL-plus reagent. Phospho-c-jun immunoblots, phospho-p38 MAPK and phospho-p44/42 were then stripped with strip buffer at 50°C for 30 min and reblotted for total c-jun, p38 MAPK and p44/42 MAPK (p44 mitogen-activated protein kinase/p42 mitogen-activated protein kinase), respectively (Cell Signaling Technology). The volume of the protein bands was quantified by a Bio-Rad ChemiDoc™ EQ densitometer and a Bio-Rad Quantity One® software (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

### SAPK/JNK kinase assay

Myocardial tissues were lysed with ice-cold lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM 1% Triton X-100, EGTA 2.5 mM, sodium pyrophosphate 1 mM, $\beta$-glycerolphosphate 1 mM, $Na_3VO_4$ 1 $\mu$g $ml^{-1}$, leupeptin and 1 mM PMSF). After sonication, lysates were centrifuged, protein concentration determined and JNK kinase activity was measured with the SAPK/JNK Assay Kit (Cell Signaling Technology) using c-jun as substrate for SAPK/JNK. Briefly, 2 $\mu$g of c-Jun fusion protein beads were added to 250 $\mu$g total protein and incubated with gentle rocking overnight at 4°C. Then samples were microcentrifuged, washed with lysis buffer and incubated with kinase buffer (25 mM Tris (pH 7.5), 5 mM $\beta$-glycerolphosphate, 2 mM DTT, 0.1 mM $Na_3VO_4$, 10 mM $MgCl_2$ and 100 $\mu$M ATP) for 30 min at 30°C. The reactions were then stopped with sample buffer and the samples loaded on SDS–PAGE gel for Western immunoblotting. The volume of the protein bands was quantified by a Bio-Rad ChemiDoc™ EQ densitometer and a Bio-Rad Quantity One® software (Bio-Rad Laboratories).

### Statistical analysis

All values in the text and figures are presented as mean ± s.e.m. of ($n$) independent experiments. All data were analyzed by one- or three-way ANOVA followed by Tukey test. $P$ values $\leq 0.05$ were considered statistically significant.

## Results

### AS601245 does not affect hemodynamics and coronary occlusion-induced ST elevation

Figure 1a–c show HR (beats $min^{-1}$), mean arterial pressure (MAP; mmHg) and pressure rate index (PRI; mmHg $min^{-1}$ $10^3$), respectively, during 30 min of coronary occlusion and 180 min of reperfusion. In ischemic control, MAP was stable throughout the experiment, a similar trend being observed for PRI. Neither 3-AB nor AS601245 administration during coronary occlusion and reperfusion affected HR, MAP and PRI when compared to saline-treated controls. Figure 1d shows the mean changes of ST segment measured during ischemia and reperfusion. ST-segment elevation values were represented as variations *versus* the respective preocclusion values. In all groups, preocclusion ST-segment values were similar. Coronary occlusion resulted in marked ST-segment elevation generally peaking after 10 min of coronary occlusion and remaining at a sustained level as long as occlusion was maintained. When reperfusion was allowed, ST-segment values progressively returned towards preocclusion levels. No significant differences among groups were found at any time.

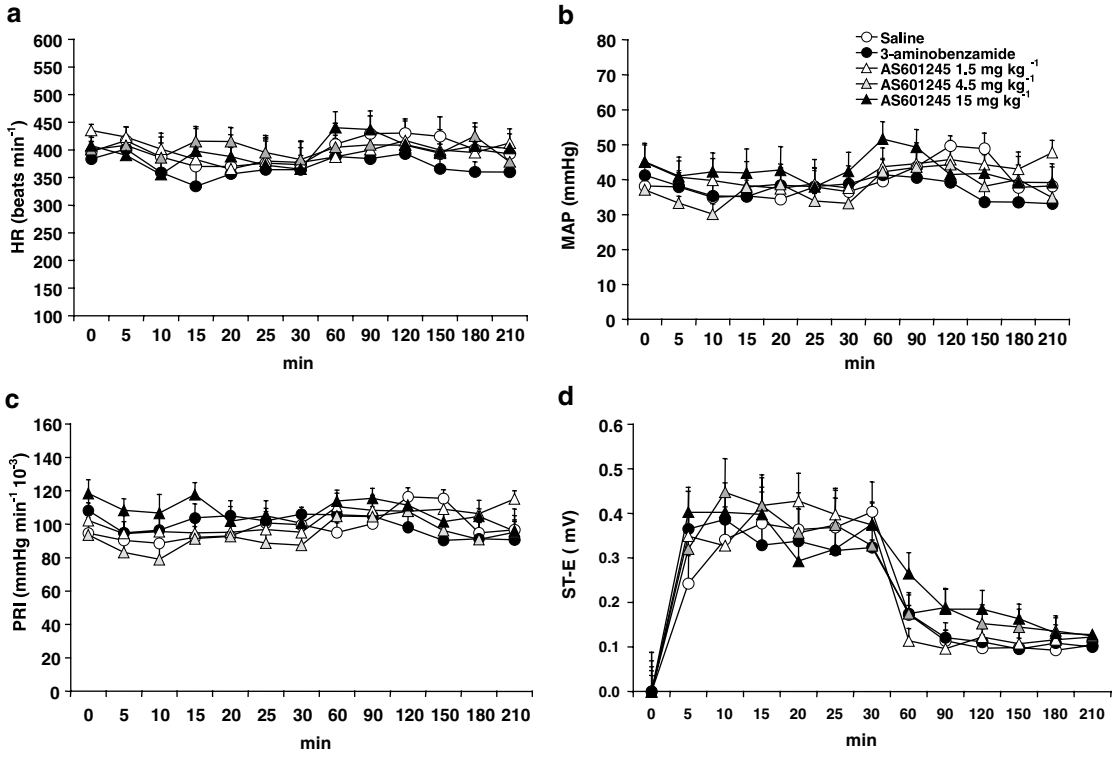


**Figure 1** Hemodynamics and ECG. Heart rate (HR; a), mean arterial pressure (MAP; b), and ECG (d) were continuously recorded throughout the experiment. Pressure rate index (PRI; c) is an index of oxygen consumption, which was calculated as the product of HR and MAP. Each point represents the mean + s.e.m. of eight separate experiments.

### *AS601245 is able to reduce IS*

In all experimental groups, the mean AAR values were similar, ranging from $50.7 \pm 2$ to $57.8 \pm 4\%$ of left ventricle (Figure 2a). In control ischemic rats, the IS was $74.1 \pm 5\%$ of AAR. In the groups receiving 3-AB or AS601245 at 1.5, 4.5 and 15 mg kg$^{-1}$ i.v., no significant difference was found in the AAR, while a statistically significant decrease ($P<0.001$) in IS was observed with 3-AB (−44.2%) and AS601245 at 4.5 (−44%) or 15 mg kg$^{-1}$ i.v. (−40.3%) in comparison to saline-treated controls (Figure 2b).

### *Administration of AS601245 decreases c-jun phosphorylation (Ser 73) in cardiomyocytes*

Pathological alterations were examined by light microscopy of hematoxylin and eosin-stained sections. No lesions were noted in myocardial sections obtained from sham-operated rats (Figure 3a), while in rats subjected to coronary occlusion followed by reperfusion in the absence (Figure 3b) and in the presence of AS601245 at 4.5 mg kg$^{-1}$ i.v. (Figure 3c), necrosis and cellular damage with perivascular edema were present mainly in the border area. Since the study's primary goal was to evaluate the effect of a JNK inhibitor on apoptosis-related biochemical and morphological alterations, the extent of necrosis was not determined.

Immunohistochemistry staining of total-c-jun in cryosectioned ischemic hearts treated with AS601245 at 4.5 mg kg$^{-1}$ i.v. showed that c-jun is expressed equally in infarct, border and non-infarct tissues of sham (Figure 3d), AS601245-treated (Figure 3e) and untreated post-ischemic rats (Figure 3f),

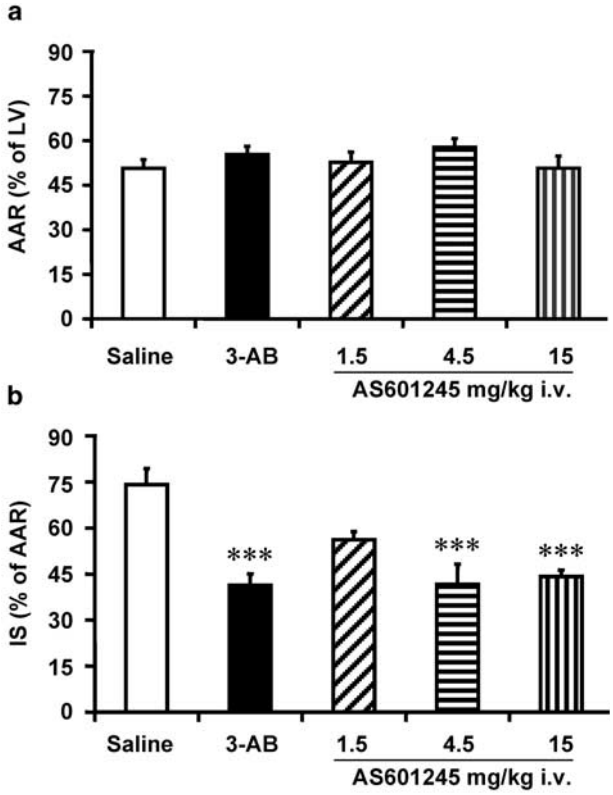


**Figure 2** Area at risk (AAR) and infarct size (IS). The IS is expressed as percentage of AAR. Each point represents the mean ± s.e.m. of eight separate experiments. ***$P<0.001$ *versus* vehicle.

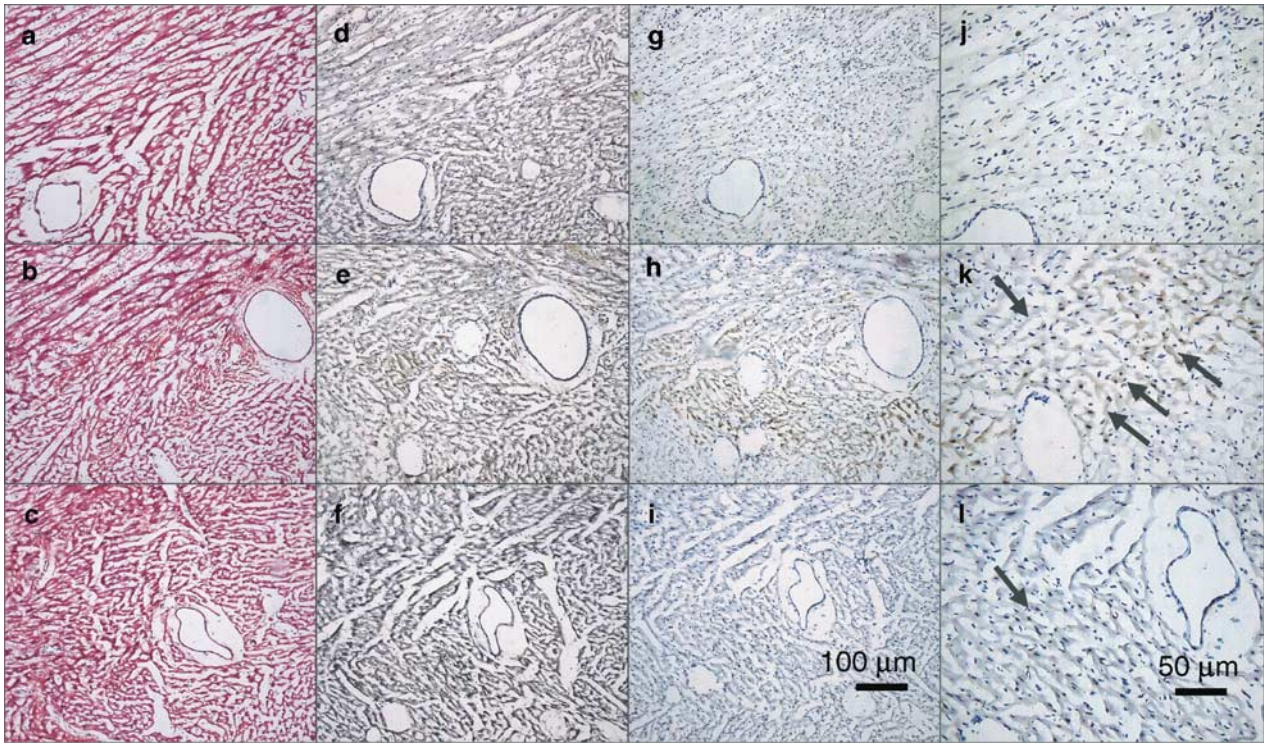


**Figure 3** Hematoxylin and eosin staining (a–c), immunohistochemical localization of c-jun (d–f) and phospho-c-jun (g–l) in sham-operated rats (a; d; g; j), in rats exposed to 30 min of ischemia followed by 3 h of reperfusion in absence (b; e; h; k) or presence of AS601245 at 4.5 mg kg$^{-1}$ i.v. (c; f; i; l). Arrowheads indicate positive nuclei for phospho-c-jun. Magnification $\times 100$ (a–i); magnification $\times 200$ (j–l).

and is localized at cytoplasmatic and nuclear levels. In contrast, immunohistochemistry detection of nuclear phospho-c-jun showed that there is a correlation of phosho-c-jun staining in infarction area both in treated and untreated post-ischemic hearts. However, AS601245 administration significantly decreases c-jun phosphorylation (Ser 73) (−85%; $P<0.001$ *versus* controls; Figures 3i,l and 4), which was marked and widespread in ischemic controls (Figure 3h, j) and completely absent in sham-operated control rats (Figure 3g, j).

### *AS601245 reduces myocardial apoptosis and inhibits DNA ladder formation*

In myocardial tissue from sham-operated rats, no DNA ladder formation (Figure 5B) and a very low level of TUNEL-positive staining (Figure 5Aa–b and 5C) were detected. TUNEL-positive cells were present only in infarct regions and not in the border area. DNA ladder formation and a significant number of TUNEL-positive cells were observed in the infarct myocardial tissue from ischemic controls (Figure 5Ac–d and B–C). AS601245 at 4.5 mg kg$^{-1}$ i.v. exerted a significant anti-apoptotic effect, as evidenced by the lower incidence of DNA ladder formation and decreased TUNEL-positive staining in the infarct region (−82.1%; $P<0.001$ *versus* controls; Figure 5Ae–f and B–C).

### *Effect of AS601245 on SAPK/JNK activation*

The level of JNK activation in hearts in response to ischemia and reperfusion was examined using three additional groups

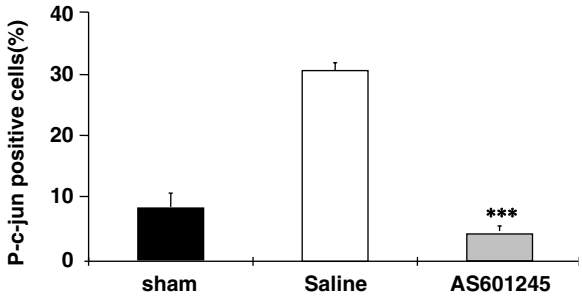


**Figure 4** Immunohistochemistry analysis of c-jun phosphorylation (Ser 73). Percentage of nuclei with positive staining for phospho-c-jun (Ser 73) in sham-operated rats or rats exposed to 30 min of ischemia followed by 3 h of reperfusion in absence or presence of AS601245 at 4.5 mg kg$^{-1}$ i.v. Each bar represents the mean ± s.e.m. of three separate experiments. ***$P<0.001$ *versus* saline-treated control group.

($n=3$ group$^{-1}$). Increased JNK activation was observed in the post-ischemic/reperfused hearts, compared to sham-operated ones. Administration of AS601245 at 4.5 mg kg$^{-1}$ i.v. was able to reduce JNK activation to baseline values. A representative assay of JNK activity is shown in Figure 6a.

### *Selectivity of the anti-ischemic action of AS601245*

JNK protein kinases are activated by phosphorylation of SEK1/MKK4, a MAPKK, and after this activation JNK is able to phosphorylate the transcription factor c-jun on two phosphorylation sites (Ser-63 and Ser 73), which causes

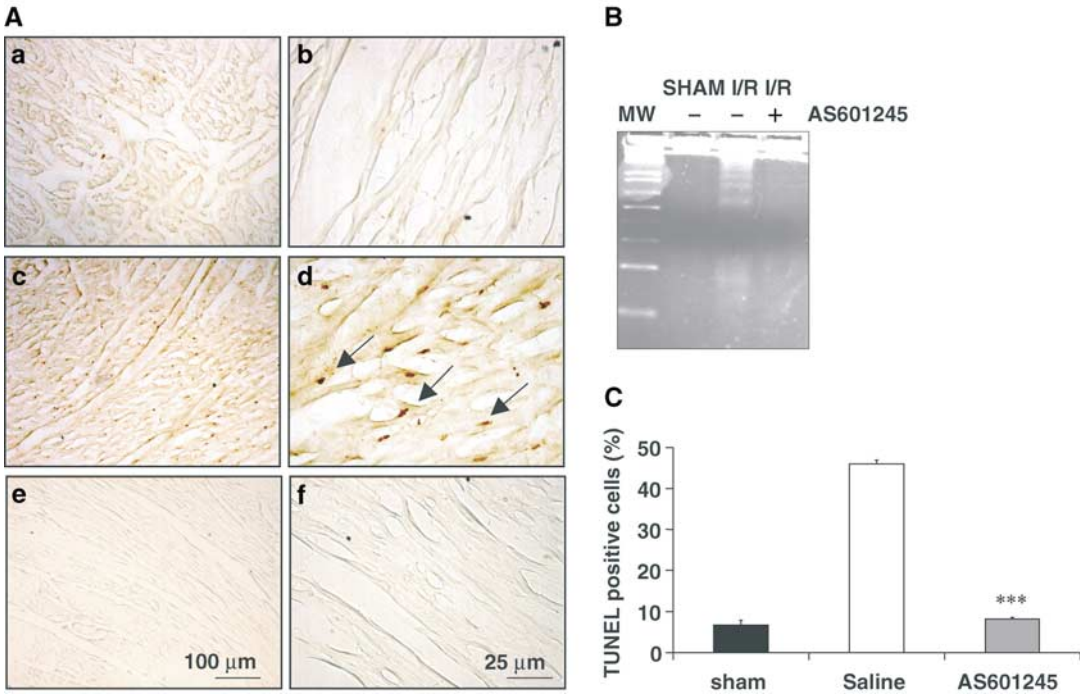


**Figure 5** (A) Representative photomicrographs of *in situ* detection of DNA fragments from sham-operated rats (a–b) or rats subjected to 30 min of ischemia followed by 3 h of reperfusion in absence(c–d) or in presence of AS601245 at 4.5 mg kg$^{-1}$ i.v. (e–f). Arrowheads indicate positive nuclei for TUNEL staining; magnification $\times 100$ (a, c, e ); magnification $\times 400$ (b, d, f). (B) Electrophoretic pattern of DNA laddering in sham-operated rats or rats subjected to ischemia followed by reperfusion in absence or in presence of AS601245 at 4.5 mg kg$^{-1}$ i.v.; I/R: ischemia/reperfusion. The panel is representative of three separate experiments. (C) TUNEL. Percentage of nuclei positively stained for TUNEL in sham-operated rats or rats exposed to 30 min of ischemia followed by 3 h of reperfusion in absence or presence of AS601245 at 4.5 mg kg$^{-1}$ i.v. Each bar represents the mean ± s.e.m. of three separate experiments. ***$P<0.001$ *versus* saline-treated control group.

increased transcription activity and apoptosis. A Western blot analysis of post-ischemic hearts was performed on the JNK signaling pathway in sham and when subjected to ischemia/reperfusion in absence and in presence of AS601245 at 4.5 mg kg$^{-1}$ i.v. As expected, AS601245 blocked c-jun (Ser 73) phosphorylation, but was unable to inhibit phosphorylation of the upstream part of the cascade (Figure 6b). In fact, phosphorylation states of SEK1/MKK4 and JNK were unaffected by AS601245, both kinases being activated by the stress conditions related to the myocardial ischemia/reperfusion.

### *Effect of AS601245 on MAPK activated during myocardial ischemia and reperfusion*

Since p38 MAPK is involved in myocardial injury caused by ischemia/reperfusion, a Western blot analysis was performed in post-ischemic hearts to investigate whether AS601245 treatment at the most effective dose (4.5 mg kg$^{-1}$ i.v.) could inhibit p38MAPK phosphorylation. After ischemia/reperfusion, AS601245 was not able to modify p38MAPK phosphorylation state (Figure 6c). Figure 6d shows the effect of AS601245 administration, at 4.5 mg kg$^{-1}$ i.v., on ERK phosphorylation (p44/42 MAPK). ERK is a kinase involved in protecting cells from apoptotic death and consistently activated after ischemia and reperfusion in the induction of proto-oncogenes and cardioprotective genes (Aikawa *et al*., 1997). AS601245 was unable to decrease the phosphorylation state of ERK as the expression of the total protein after ischemia and reperfusion.

## Discussion

This study reports for the first time that a pharmacological intervention aimed at inhibiting myocardial ischemia/reperfusion-induced JNK activation exerts a beneficial effect in reducing cardiomyocyte apoptosis and death. A strong correlation between JNK activation and apoptosis induction has been described in the heart and kidney exposed to ischemia/reperfusion (Yin *et al*., 1997; Armstrong, 2004). Cook *et al*. (1999) reported enhanced activation of JNK and p38 MAP kinases in hearts from patients with heart failure caused by ischemic disease, suggesting that activation of these kinases may contribute to the pathophysiology of the disease.

AS601245, a specific nonisoform selective JNK inhibitor belonging to a new class of benzothiazole derivatives, significantly reduced IS after coronary occlusion and reperfusion in anaesthetized rats. This protective effect was obtained without affecting hemodynamics or reducing ST-segment displacement and was apparently due to inhibition of ischemia/reperfusion-induced apoptosis. After prolonged ischemia, cardiomyocyte cell death is attributable to necrosis, while apoptosis occurs in cells and tissues exposed to reoxygenation after ischemia (Hreniuk *et al*., 2001). It has been also demonstrated in rat heart that JNK translocates to

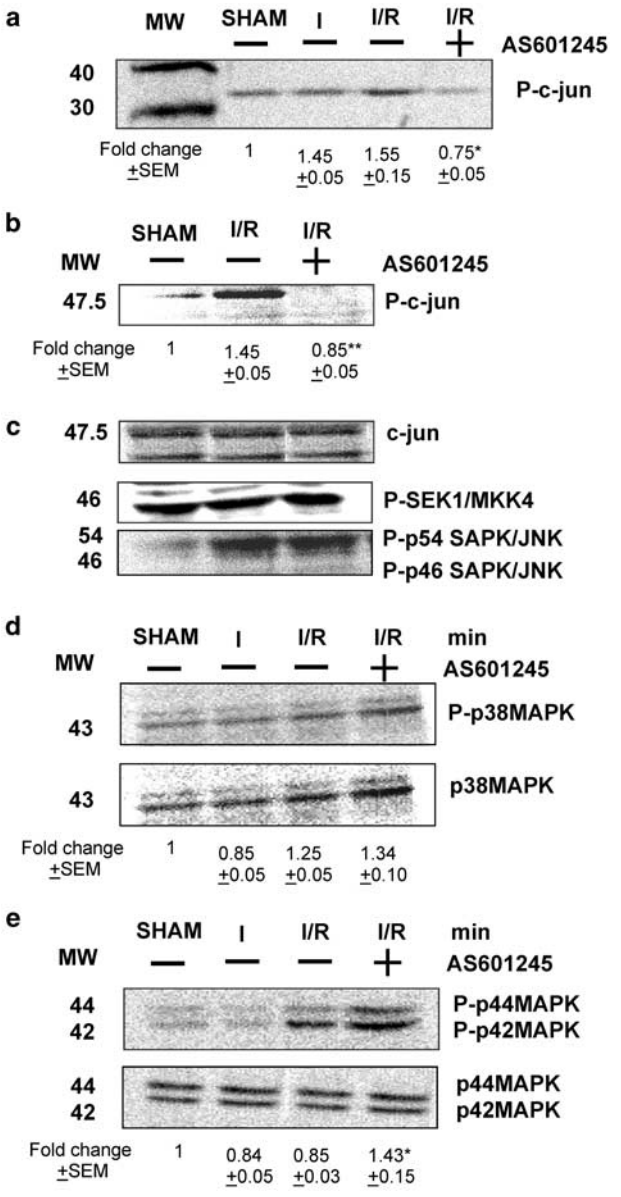


**Figure 6** Biochemical investigation on postischemic hearts to assess the selectivity of the anti-ischemic properties of AS601245. Panels are representative of three separate experiments performed in sham-operated rats, in rats subjected to 30 min of ischemia (I) and rats subjected to 30 min of ischemia followed by 180 min of reperfusion (I/R) in absence or in presence of AS601245 at 4.5 mg kg$^{-1}$ i.v. Quantitative analysis is expressed as fold change over the sham group. *$P<0.05$; **$P<0.01$ (a) SAPK/JNK kinase assay. (b) Western blot analysis of SAPK/JNK signaling cascade. (c) Western blot analysis of phospho-p38 MAPK and total p38 MAPK. (d) Western blot analysis of phospho-p44/42 MAPK and total p44/42 MAPK.

the nucleus during ischemia to be phosphorylated during the reperfusion period, a process able to activate the apoptotic signaling pathway. In our results, 30-min ischemia followed by 3-h reperfusion produced a high level of apoptosis shown by terminal nick-end labeling of DNA fragmentation and DNA oligonucleosomal laddering. After treatment with AS601245, this active gene-directed process was fully blocked. These results demonstrate that inhibition of JNK activity and subsequent reduction of ischemia/reperfusion myocardial-induced apoptosis are able to improve cardiac functions in post-ischemic hearts.

Another important finding is that administration of AS601245 during reperfusion completely blocked the JNK pathway by inhibiting c-jun phosphorylation in infarct tissues. A pronounced decrease was seen in the phosphorylation state of its downstream target c-jun (Ser 73). On the contrary, the phosphorylation state of JNK itself, as well as of the related signaling proteins like SEK1/MKK4, remained unchanged in the presence of AS601245. We also demonstrated a clear activation of the two other major MAP kinase pathways at the end of the post-ischemic reperfusion phase by measuring the phosphorylation states of p38 and ERK, which once again remained unchanged following treatment with AS601245. Although we cannot rule out the fact, due to the ATP nature of the inhibitor, a number of existing kinases and potential inhibition of other signaling pathways might be involved. These results clearly demonstrate that the cardiovascular protection observed with AS601245 is related to JNK phosphorylation blockade.

However, it would be useful to identify the roles of the different JNK isoforms in ischemia/reperfusion injury, given their differing specificity for downstream transcription factors (Gupta *et al*., 1996) as well as in their stress-induced activation (Butterfield *et al*., 1999). While JNK1 and JNK2 are widely expressed, JNK3 is predominantly found in brain and to a lesser extent, in heart and testis. Hreniuk *et al*. (2001) showed that JNK1 isoform plays a preferential role in apoptosis induced by ischemia/reoxygenation. However, since AS601245 is not specific for any JNK isoform this, in turn, may be advantageous in maximizing its pharmacological effect, and inhibition of the three isoforms may prevent possible isoform compensation during the course of ischemic injury.

Reduction of IS by AS601245 increased with the dose, but significant differences from controls were found only with the 4.5 mg kg$^{-1}$ plus 55 $\mu$g kg$^{-1}$ min$^{-1}$ and 15 mg kg$^{-1}$ plus 183 $\mu$g kg$^{-1}$ min$^{-1}$ dose regimens, at expected plasma levels of 3 and 10 $\mu$g ml$^{-1}$. The protective activity of AS601245 plateaued at 4.5 mg kg$^{-1}$ plus 55 $\mu$g kg$^{-1}$ min$^{-1}$. No further reduction of IS was achieved with the highest dose, very likely due to possible saturation of ATP binding site of JNK, reaching therefore a maximal effect. However, interactions of AS601245, especially at high doses, with other kinases could be not excluded. Finally, the plasma concentration (3 $\mu$g ml$^{-1}$ corresponding to a concentration of 5 $\mu$M) at which AS601245 significantly decreased IS, as well as cardiomyocyte apoptosis and JNK activation in post-ischemic heart, is very close to those exerting antiapoptotic effects in cell cultures (Pascale Gaillard, personal communication), suggesting that JNK inhibition is responsible for the antiapoptotic effects of AS601245, reducing the severity of infarction.

In summary, treatment with the novel JNK inhibitor AS601245 during myocardial ischemia and reperfusion significantly reduces myocardial apoptosis associated with JNK activation and improves postischemic cardiac functional recovery. These results are potentially of clinical significance, and may suggest a new therapeutic strategy for treating ischemic heart disease.

We are grateful to Flavio Boin, Mauro Giachetto and Diego Bignotti for their expert and precious technical support.

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*(Received March 22, 2004*
*Revised May 4, 2004*
*Accepted May 10, 2004)*