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Oxidant-induced cardiomyocyte injury: Identification of the cytoprotective effect of a dopamine 1 receptor agonist using a cell-based high-throughput assay

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Abstract. Myocyte injury due to myocardial reperfusion injury plays a crucial role in the pathogenesis of acute myocardial infarction even after successful coronary revascularization. Identification of compounds that reduce reperfusion-associated myocyte death is important. Therefore, we developed an *in vitro* model of myocardial reperfusion injury in H9c2 rat cardiomyocytes and applied a cell-based high-throughput approach to screen a standard library of pharmacologically active compounds (LOPAC) in order to identify drugs with cardioprotective effects. Oxidative stress was induced with hydrogen peroxide (H_2O_2) treatment, which resulted in ~50% reduction in cell viability. Test compounds were added at a 3-μM final concentration as a pretreatment or in a delayed fashion (30 min after the peroxide challenge in order to imitate pharmacological treatment following angioplasty). Cells were cultured for 3 or 24 h. Viability was quantitated with the methylthiazolyldiphenyl-tetrazolium bromide method. Cytotoxicity and cytoprotection were also evaluated by measuring the lactate dehydrogenase activity in the cell culture supernatant. The screening identified a number of compounds with cytoprotective action, including molecules that are known to interfere with components of DNA repair and cell cycle progression, e.g. poly(ADP-ribose) polymerase (PARP) inhibitors, topoisomerase inhibitors, and cyclin dependent kinase inhibitors, or reduce energy consumption by interfering with cardiac myofilament function. A number of dopamine D1 receptor agonists also provided significant cytoprotection at 3 h, but only three of them showed a similar effect at 24 h: chloro- and bromo-APB and chloro-PB hydrobromide. Chloro-APB hydrobromide significantly reduced peroxide-induced PARP activation in the myocytes independently of its action on dopamine D1 receptors, but lacked PARP inhibitor capacity in a cell-free PARP assay system. In conclusion, the pattern of cytoprotective drugs identified in the current assay supports the overall validity of our model system. The findings demonstrate that cytoprotective agents, including novel indirect inhibitors of cellular PARP activation can be identified with the method, chloro-APB hydrobromide being one such compound. The current experimental setting can be employed for cell-based high-throughput screening of various compound libraries.

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Abbreviations: APB, 7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; LDH, lactate dehydrogenase; LOPAC, library of pharmacologically active compounds; MTT, methylthiazolyldiphenyl-tetrazolium bromide; PAR, poly(ADP-ribose); PARP, poly(ADP-ribose) polymerase; PBS, phosphate buffered saline; PJ34, N-(-oxo-5,6-dihydro-phenanthridin-2-yl)-N,N-dimethylacetamide HCl

Key words: cell necrosis, apoptosis, oxidative stress, poly(ADP-ribose) polymerase, myocardial infarction, high-throughput screening, high content screening

Introduction

Cardiomyocyte injury (necrosis and apoptosis) plays a crucial role in the pathogenesis of acute myocardial infarction during the ischemic phase. There is also a secondary myocyte injury (termed 'reperfusion injury') in conjunction with successful coronary revascularization, evidenced by a secondary elevation of plasma enzymes indicative of myocyte injury (1,2). Identification of compounds that reduce reperfusion-associated myocyte death, therefore, is important, and a number of drugs that have entered clinical trials were aimed at attenuation of myocyte reperfusion injury (3-6).

The pathogenesis of reperfusion injury of the heart is complex, but it is frequently modeled *in vitro* using ischemic and reoxygenated perfused hearts. Here we developed an *in vitro* model of myocardial reperfusion injury in H9c2 rat cardiomyocytes and applied a cell-based high-throughput approach to screen a standard library of pharmacologically active compounds (LOPAC) in order to identify compounds with cardioprotective effects.

As there is clear evidence that the generation of reactive oxygen and nitrogen species plays a primary role in the

pathogenesis of myocardial reperfusion injury (7-10), cell death was induced by hydrogen peroxide treatment. In one set of screening experiments, the compounds were added in a pretreatment regimen. In a subsequent set of experiments, the time of the administration of the compounds to be screened was delayed relative to the hydrogen peroxide treatment, in order to simulate a clinically more relevant situation, and also to avoid the identification of antioxidant compounds that directly interfere with the primary effect of hydrogen peroxide in inducing cell injury. Since activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) plays a crucial role in myocyte death during coronary ischemia-reperfusion (11-15), a potent PARP inhibitor was included in the assays as a positive control.

A number of compounds, including several dopamine D1 receptor agonists emerged from the assays as significant cytoprotective agents; the mechanism of the protective action of these compounds was therefore subsequently investigated in more detail.

Materials and methods

Generic library. A library of 1280 pharmacologically active compounds (LOPAC1280) was obtained from Sigma-Aldrich. The library includes drug-like molecules in the field of cell signaling and neuroscience. The compounds were dissolved at 10 mM in dimethyl sulfoxide (DMSO) and dilutions were made either in DMSO or phosphate-buffered saline (PBS, pH 7.4) to obtain 0.5% DMSO in the assay volume. The potent poly-ADP-ribose polymerase (PARP) inhibitor N-(-oxo-5,6-dihydro-phenanthridin-2-yl)-N,N-dimethylacetamide HCl (PJ34) (16) was purchased from Calbiochem. Chloro-APB (6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine) hydrobromide and Bromo-APB (6-bromo-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine) hydrobromide were from Sigma. These compounds were dissolved in DMSO for the cell-based screen or in ultrapure water for the PARP assays.

Cell culture. H9c2 rat heart myoblast cells were obtained from the European Collection of Cell Cultures and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen), 4 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Seven days prior to the screening assay 10,000 cells/well were plated into 96-well tissue culture plates and cultured at 37˚C in a 5% CO_2 atmosphere. Cardiomyoblasts from passage numbers 40-60 were used for the screening. For the measurement of PARP activity, cells were plated into 60-mm culture dishes and cultured until confluence.

MTT viability assay. Fresh culture medium without phenol-red was added to the cells prior to the assay, and cardiomyoblasts were challenged with 900-1100 μM H_2O_2. In the standard assays, 30 min later, drugs were added at a 3-μM concentration in 5% of the culture volume and cultured at 37˚C for 3 or 24 h. In pretreatment assays cells were pre-incubated with drugs for 30 min prior to the addition of H_2O_2 and incubated with peroxide for 3.5 or 24.5 h. The supernatant (30 μl) was saved for the measurement of the lactate dehydrogenase (LDH) release and stored at -20˚C until assayed. To estimate the number of viable cells 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added to the cells at a final concentration of 0.5 mg/ml and cultured at 37˚C for 1 h (16,17). Cells were washed with PBS, and the formazan dye was dissolved in isopropanol. The amount of converted formazan dye was measured at 570 nm with background measurement at 690 nm on a Powerwave reader (Biotek). A calibration curve was created by measuring the MTT converting capacity of serial dilutions of H9c2 cells, and the viable cell count was calculated using Gen5 data reduction software.

LDH assay. Cell culture supernatant (30 μl) was mixed with 100 μl freshly prepared LDH assay reagent to reach final concentrations of 85 mM lactic acid, 1040 mM nicotinamide adenine dinucleotide (NAD), 224 mM *N*-methylphenazonium methyl sulfate (PMS), 528 mM 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride (INT) and 200 mM Tris (pH 8.2). The changes in absorbance were either read kinetically at 492 nm for 15 min (kinetic LDH assay) or plates were incubated for 15 min and read at 492 nm with background measurement at 690 nm (endpoint assay) on a monochromator based reader (Powerwave HT, Biotek). LDH activity was expressed as percent values of V_{max} (for kinetic assay) or ΔOD (492-690 for endpoint assay) of control wells receiving hydrogen peroxide (100%), with controls receiving vehicle only as blank (0%).

Measurement of PARP activity by PAR Western blot analysis. Confluent cultures of H9c2 cells in 60-mm dishes were washed with serum-free medium and pretreated with drugs or vehicle for 30 min, and then cells were challenged with 500 μM H_2O_2 (Sigma) for 60 min. After three washes with ice-cold phosphate-buffered saline (PBS, pH 7.4), cells were lysed in 400 μl denaturing loading buffer (20 mM Tris, 2% SDS, 10% glycerol, 6 M urea, 100 μg/ml bromophenol blue, 200 mM ß-mercaptoethanol), sonicated and boiled. Lysates (10 μl) were resolved on 4-12% NuPage Bis-Tris acrylamide gels (Novex, Invitrogen) and transferred to nitrocellulose. Membranes were blocked in 10% non-fat dried milk and probed overnight with anti-poly(ADP-ribose) antibody (anti-PAR, 1:2000, Calbiochem). Anti-rabbit-horseradish peroxidase conjugate (HRP, 1:2000, Cell Signaling) and enhanced chemiluminescent substrate (ECL, Pierce) were used to detect the chemiluminescent signal in a CCD-camera-based chemiluminescence detection system (Genegnome HR, Syngene). To normalize signals, membranes were re-probed with an antibody against α-tubulin. Briefly, membranes were stripped in 62.5 mM Tris, 2% SDS, 100 mM ß-mercaptoethanol at 60˚C for 30 min, blocked overnight in 5% non-fat dried milk and re-probed with anti-tubulin (1:6000, Sigma) monoclonal antibody. After the application of anti-mouse-HRP conjugate (1:10 000, Cell Signaling) and ECL, chemiluminescence was detected with the same imaging system. The PAR signal at 116 kDa, which corresponds to the automodified form of PARP-1, and the chemiluminescent signal at ~50 kDa for tubulin were quantitated with Genetools analysis software, and their ratio was expressed as relative PARP activity.

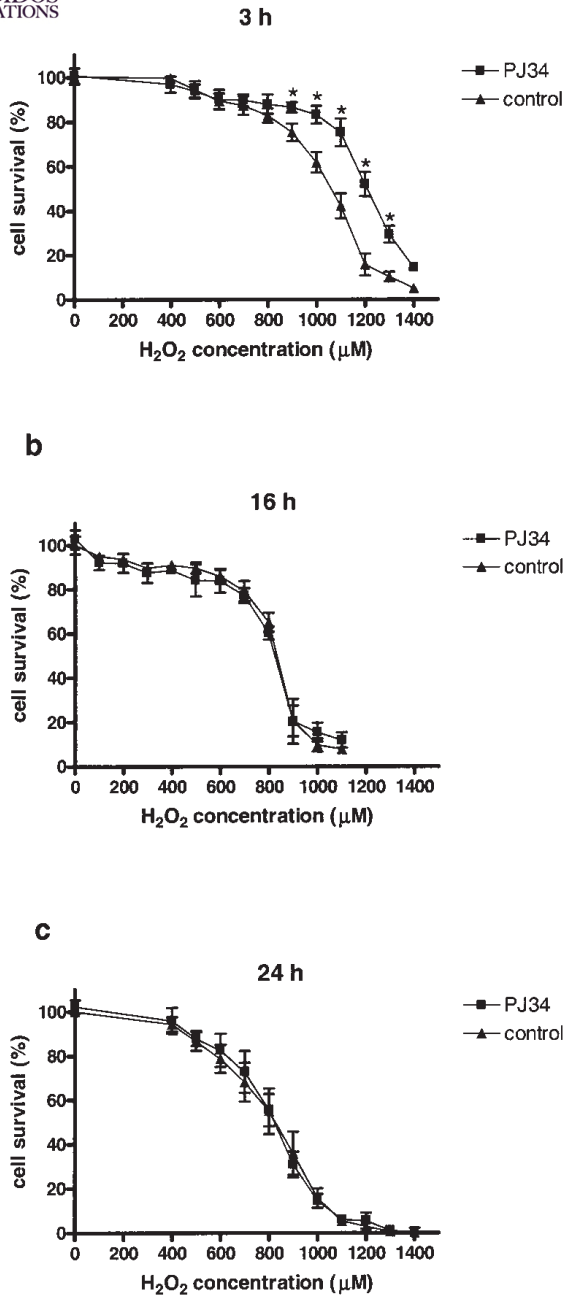

Figure 1. H_2O_2-induced cytotoxicity in the absence or presence of the PARP inhibitor PJ34. Percent survival values at 3 (a), 16 (b) and 24 h (c) are shown as the mean ± SEM. Controls received increasing concentrations of H_2O_2, and PJ34-treated cells received PJ34 at a 3-μM final concentration, 30 min after the H_2O_2 treatment. Data are shown as the mean ± SEM ($^*p<0.05$).

Cell-free colorimetric PARP activity assay. PARP activity was measured with a commercial PARP assay kit according to the protocol provided by the manufacturer (R&D Systems) (16). Briefly, test compounds and 1 U PARP enzyme were added to histone-coated wells. A cocktail containing activated DNA and biotinylated-NAD was added to the wells and incubated for 60 min. After 4 washes with PBS biotinylated-ribose, residues were detected with streptavidine-HRP conjugate and TACS-sapphire substrate. After 30 min the reaction was stopped with 0.2 M HCl, and absorbance values were measured at 450 nm.

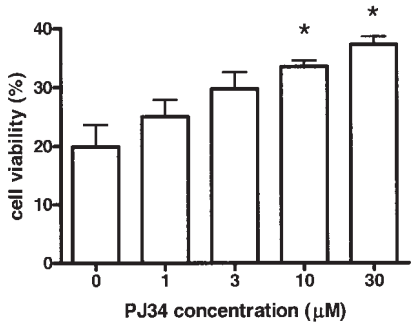

Figure 2. PARP inhibitor induced cytoprotection. H9c2 cardiomyoblasts were treated with H_2O_2 (850 μM) and various concentrations of PJ34. Cell viability was quantitated with MTT assay after 24 h. Viable cell counts are expressed as percent values of vehicle-treated cells. Data are shown as the mean ± SEM ($^*p<0.05$).

Results and discussion

Establishment of the oxidant-induced cardiac cell death model. We aimed to achieve a reproducible myocyte cell death model, which can be used in cell-based screening for compounds that exhibit cytoprotective effects. The H9c2 rat cardiomyoblast cell line was chosen to provide a source of cells with a well-characterized phenotype. Cells were cultured for 7 days prior to the assay to reach a more differentiated state similar to mature cardiomyocytes. When cells were treated with increasing concentrations of H_2O_2 for 3 h, a dose-dependent reduction was detected in cell viability (Fig. 1a) using the MTT assay. The reduction in viable cell count was >25% for up to a 900-μM final concentration of H_2O_2. When elevating the concentration of hydrogen peroxide by an additional 300-μM range (to reach a 1.2-mM concentration), the viable cell count rapidly decreased to near zero. The sensitivity of the cells to the toxic effect of H_2O_2 decreased with the number of initial cell count and passage number (data not shown). Pretreatment with the potent poly(ADP-ribose)-polymerase (PARP) inhibitor PJ34 (3 μM) for 30 min significantly increased the number of viable cells at concentrations that caused >20% reduction in cell viability. The results were similar when PJ34 was added 30 min after the H_2O_2 treatment and cell viability was quantitated 3 h after the addition of the PARP inhibitor; ~70% cell viability was measured for the PJ34-treated cells at peroxide concentrations that caused 60% reduction in the vehicle-treated group. When the incubation time was increased to 16 or 24 h, the tolerated H_2O_2 concentration decreased to <700 μM and the protective effect of PJ34 (3 μM) observed at 3 h was completely abolished (Fig. 1b and c). However, PJ34 resulted in a significant increase in viability at 24 h when applied at higher concentrations (Fig. 2).

Based on the above findings, cells were treated with hydrogen peroxide to attain ~50% cell death after 3 h (short-term survival) or 24 h (long-term survival) of incubation. Test compounds were added either 30 min prior to or 30 min after the application of hydrogen peroxide (Fig. 3). All our assay plates included negative controls that remained untreated (CTL3), positive controls that were treated with peroxide only (CTL1) and hydrogen peroxide-treated cells which also

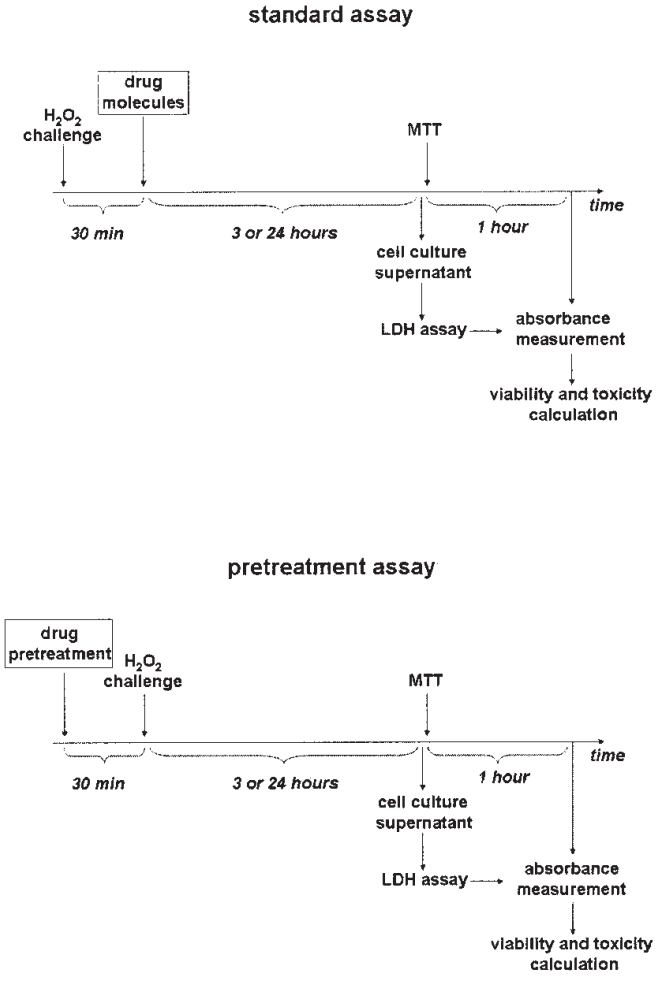

Figure 3. Experimental design. In standard assays treatment with the test compounds followed H_2O_2 challenge by 30 min, whereas in the pretreatment assays cells received test compound treatment 30 min prior to the peroxide challenge. Incubation periods of 3 and 34 h were applied in both assay types, followed by the MTT viability assay, which lasted for 1 h.

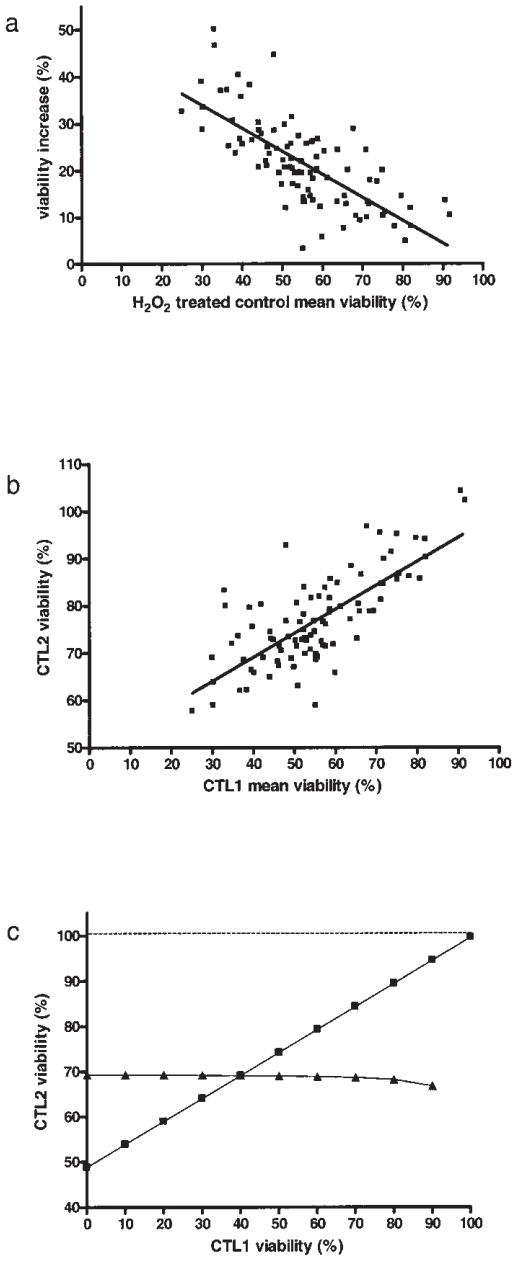

Figure 4. PARP inhibitor-induced cytoprotection at various degrees of oxidant-induced cell death. PJ34-induced mean viability increase (a) and absolute viability (b) values are shown at various degrees of H_2O_2-induced cell survival (CTL1 viability) from 100 independent experiments. Data are shown as percent values of the vehicle-treated controls. The linear correlation graph (a) equation was used to normalize H_2O_2 and PJ34-treated cell (CTL2) viability data. Expected (■) and normalized (▲) viability data are shown (c); the x-axis represents CTL1 percent viability.

received the PARP inhibitor PJ34 as a pharmacological positive control (CTL2). The relatively narrow range of H_2O_2 that caused 50% reduction in cell viability and the variability of cell counts observed 7 days after plating at different passage numbers often resulted in variable cell survival data ranging from nearly complete cell death to insignificant reduction in viability at a given peroxide concentration. To make the measurements taken on separate plates comparable, we applied a normalization of the viability data, which presumed a 40% normalized cell survival in the peroxide treated controls and a 100% survival for untreated controls by using the following equation:

$$\text{Normalized viability} = \frac{60}{100 - \text{mean (CTL1)}} \times \text{Measured viability} + 100 \times \left(1 - \frac{60}{100 - \text{mean (CTL1)}}\right)$$

The feasibility of linear transformation of viability data was supported by the reproducibility of normalized viability values received for PARP inhibitor-treated controls over a wide range of viability values. Mean viability increase values for the PJ34-treated controls from 100 randomly selected plates were plotted over the measured mean viability values of peroxide-treated controls (Fig. 4). Fig. 4c shows that using this equation to predict viability data of the PJ34-treated controls resulted in <1% variability in the normalized values for the range of 10-70% measured cell survival of the peroxide-treated controls.

PARP activation plays a central role in oxidative stress-induced cardiomyocyte death. PARP inhibition by PJ34 dose-dependently increased the number of viable cells in the H_2O_2-induced cell death model (Fig. 2). To further clarify the role of PARP activation in this model, confluent H9c2 cultures

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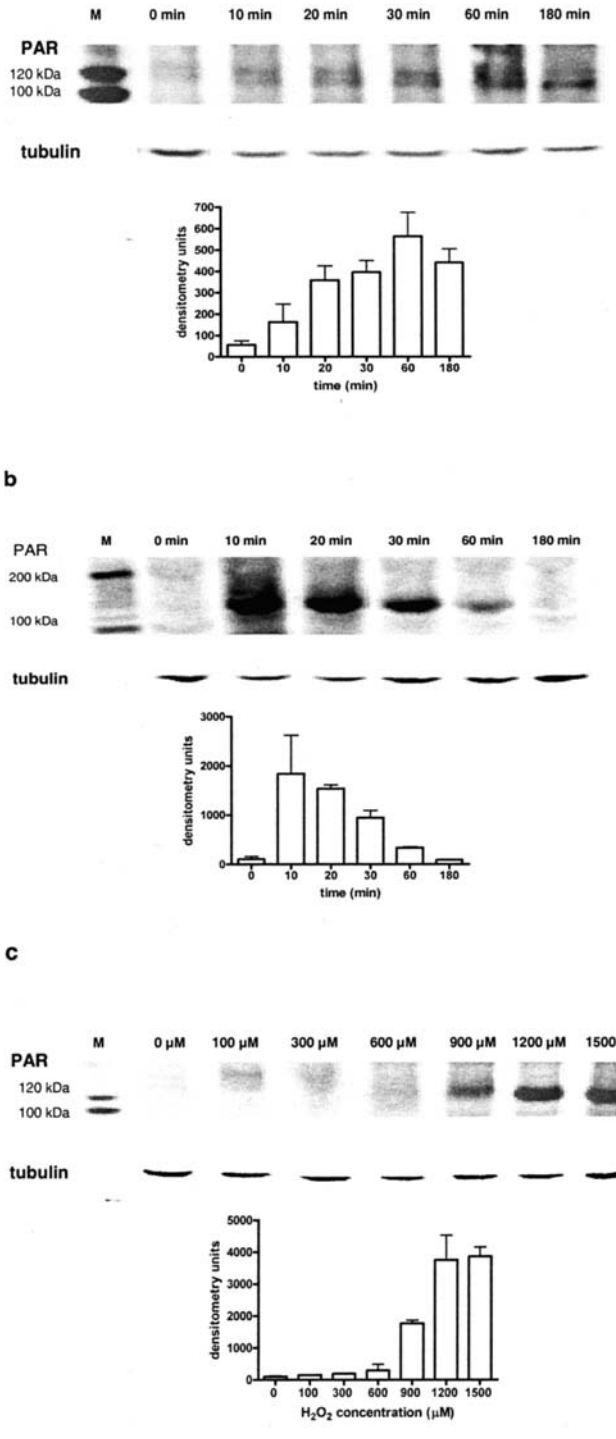


Figure 5. PARP activation in response to H_2O_2 treatment. H9c2 cells were treated with 500 μM (a) and 1 mM (b) H_2O_2 for various time points or with various concentrations of H_2O_2 for 60 min (c). PARP activation was detected as poly(ADP-ribose) polymer (PAR) formation on the Western blots. Representative blots and densitometric analysis results are shown. The 120-kDa band corresponding to PARP automodification was analysed by densitometry. PAR densitometry results were normalized to tubulin signal, and are shown as the mean ± SEM ($^*p<0.05$).

were treated with 500 μM or 1000 μM H_2O_2 for various lengths of time, and Western blotting was performed on total cell lysates for poly(ADP-ribose) (PAR), the product of PARP enzyme activity. PARP activation was detected 5 min after

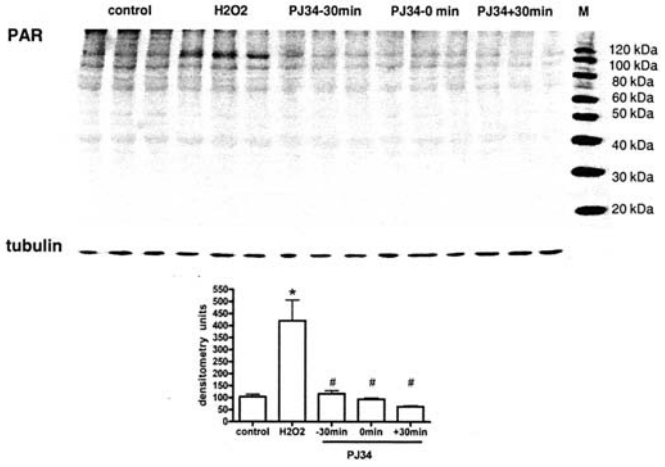


Figure 6. PJ34 blocks PAR polymer formation. PARP was activated in H9c2 cells by treating cells with H_2O_2 (500 μM) for 60 min (H_2O_2). Cells received 3 μM PJ34 30 min prior to (PJ34 - 30 min), simultaneously with (PJ34 - 0 min) or 30 min after (PJ34 + 30 min) the H_2O_2 challenge. PARP activation was detected by Western blotting. A representative blot and densitometric analysis results are shown. $^*p<0.05$ compared to the control, $^\#p<0.05$ compared to H_2O_2-treated cells.

the addition of H_2O_2 in both cases, but showed different kinetics. Maximum activity was reached only after 60 min when 500 μM H_2O_2 was used and the PAR signal remained unaltered for the third hour of incubation. H_2O_2 (1000 μM) resulted in a more robust activation, and the signal reached maximum values in 20 min followed by a decline to near baseline values at 3 h (Fig. 5a and b).

H9c2 cells were also challenged with increasing concentrations of H_2O_2 for 1 h to directly compare the PARP activation signal. A significant degree of PARP activation was detected from H_2O_2 concentrations >300 μM, with the highest values measured at >1 mM peroxide (Fig. 5c). PJ34 abolished PARP activation when 500 μM H_2O_2 was used to activate PARP, irrespective of whether it was applied 30 min earlier, simultaneously with the oxidant or 30 min later (Fig. 6). PJ34 also significantly reduced PARP activation when 1 mM H_2O_2 was applied (data not shown).

Results from the generic library screening. In the H_2O_2-induced cell death model, we used 4 different assay designs to identify compounds with cytoprotective potential; 3- and 24-h incubation periods were applied and test compounds were added either 30 min prior to or 30 min after the hydrogen peroxide challenge (Fig. 3). The following two criteria were used to identify drugs that reproducibly result in cytoprotection: i) normalized viability data were averaged for replicate plates and drugs that resulted in greater mean viability values than the average ±2 SD of all H_2O_2-treated wells, except that the PARP inhibition controls were selected as hits; ii) molecules were also selected, if the difference between the mean and the standard deviation (SD) of the normalized viability values was greater than the sum of the average viability and its standard deviation for all H_2O_2-treated sample wells (see example in Fig. 7).

Table I summarizes the hit molecules in the 3- and 24-h assays. Known PARP inhibitor compounds, with previously

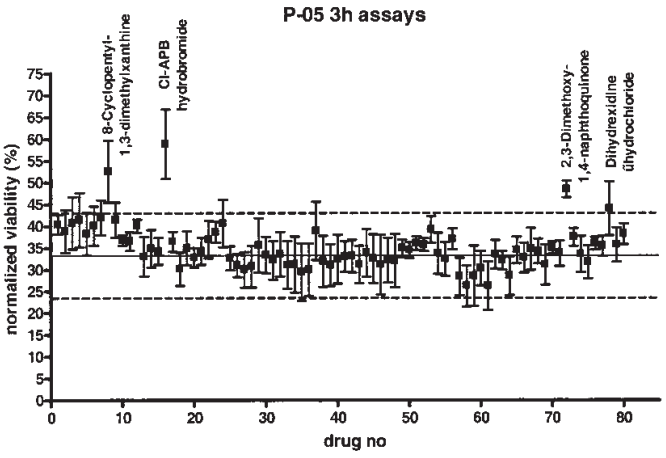


Figure 7. A representative example (normalized viability values for a series of test compounds) demonstrating the identification of Cl-APB hydrobromide as a cytoprotective compound. Cell viability values were transformed to obtain 40% viability for the average of all samples and H_2O_2-treated controls and 100% cell viability in vehicle-treated wells. The transformed viability values measured in independent experiments were averaged for each sample. Mean ± SEM values are shown. Continuous line represents the mean of all samples, while the dashed line represents the ±2 SD interval.

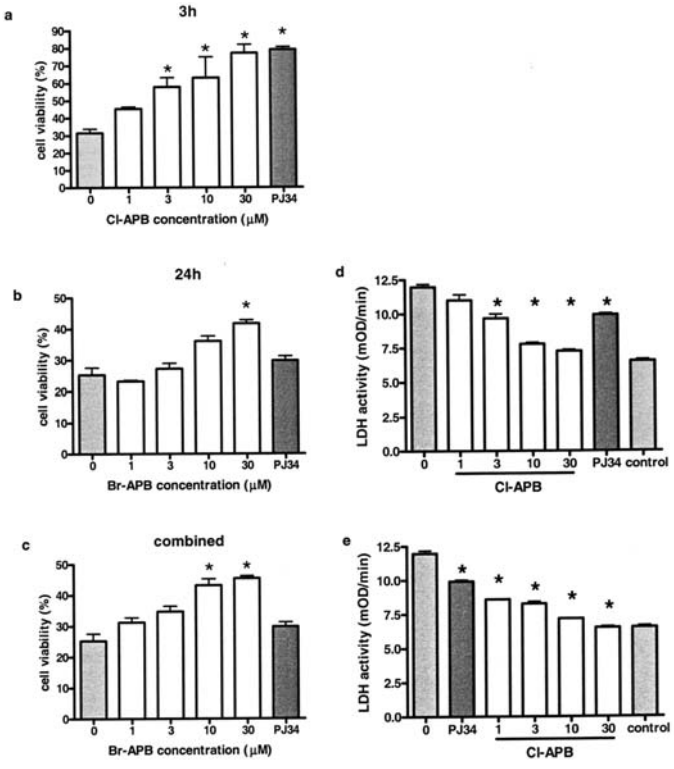


Figure 8. Cytoprotective effect of APB hydrobromide molecules. H9c2 cardiomyocytes were treated with H_2O_2 for 3 (a) or 24 h (b-e). Thirty minutes after the H_2O_2 challenge (1100 μM), Cl-APB hydrobromide (a,d,e) or Br-APB hydrobromide (b,c) was added to the cells at the indicated concentrations. The PJ34 group received 3 μM PJ34; the controls were treated with vehicle only. In the combined treatment (c,e) each sample received PJ34, except for controls. Cell viability was measured by MTT assay and expressed as percent values of controls. LDH activity in cell culture supernatant was measured kinetically and is shown in mOD/min. Mean ± SEM values are shown. $^*p<0.05$ compared to H_2O_2-treated cells.

documented cardioprotective effects (13) have reproducibly emerged as effective molecules at 3 h. These compounds also showed a trend for cytoprotection at 24 h but these effects remained under the level our selection criteria; this is consistent with the results obtained with the positive control PARP inhibitor PJ34, which also lost some of its cytoprotective potency by 24 h. Other active molecules emerging from the screen included drugs that interfere with DNA repair and cell cycle progression. Their protective effect is likely based on a general mechanism independent of cell type. Furthermore, active molecules found in our 3-h assay included ion channel blockers and cell surface receptors that were previously shown to ameliorate cell injury in oxidative stress in cultured cells (in many cases cardiomyocytes) *in vitro* or in various models of myocardial infarction and injury *in vivo* (4,6,17-28). The effects of some of these compounds can be, again, linked to the PARP pathway, as minocycline has recently been identified as a fairly potent competitive PARP inhibitor (19,20), and the protective effect of certain calcium modulators may also be linked to an early calcium-dependent step in the cellular activation of PARP (18). The cytoprotective effect of nicotine has also been demonstrated previously, and occurs, in part via inhibition of the release of the nuclear protein high mobility group protein B-1 (21). The cardioprotective effect of dipyridamole, chlorpromazine, hydrocorticosterone, forskolin and A1 agonists emerged from our screening as well: cytoprotective and/or cardioprotective effects of these compounds have also previously been described in the literature, not uncommonly with a somewhat unspecific or mixed mechanism of action (e.g. glucocorticoids are known as 'membrane stabilizing' agents). Interestingly, cholinergic agonists also showed some protective effect in 3 h, which may be the consequence of the mixed cardiomyocyte and skeletal muscle phenotype of the H9c2 cells. Overall, the identification of active molecules that act on various cell surface receptors establishes our model as a valid tool to discover novel cytoprotective drugs in a model of myocyte necrosis. Because of the experimental design employed in our standard assays (delayed treatment of the test compounds, relative to the administration of hydrogen peroxide), we did not seek to identify compounds that directly interfered with the primary oxidant injury induced by hydrogen peroxide. It is noteworthy, nevertheless, that in cultured cells and hearts exposed to a single burst of pro-oxidant challenge, a secondary, mitochondria-derived release of oxidant species also occurs (29,30). Hence, it is conceivable that some of the compounds identified herein may have attenuated this secondary oxidant response.

Most of the drugs that provided cytoprotection in 3 h were less protective in the 24-h assays. However, the cytoprotection afforded by hydrocorticosterone and forskolin was sustained in the long term. Chloro-APB hydrobromide, a D1 dopamine receptor agonist, and some closely related molecules (Bromo-APB- and Chloro-PB hydrobromide) resulted in superior cell survival values at 24 h and drastically different effects compared to other D1 dopamine receptor agonists that showed a protective effect in 3 h. As cardioprotective effects of D1 ligands have not been previously described in the literature, and yet these compounds afforded a significant degree of sustained cytoprotection in all of our assays, these compounds were selected for further study.

Cytoprotective drugs identified in rat cardiac myocytes exposed to cytotoxic concentrations of hydrogen peroxide.[a]

Name	Action	Mean viability increase (%)			
		3 h		24 h	
		ST	PRE	ST	PRE
Cell cycle: DNA repair and DNA synthesis inhibitors, cyclin-dependent protein kinase inhibitors					
4-Amino-1,8-naphthalimide	PARP inhibition	25	20		
1,5-Isoquinolinediol		12	12		
Benzamide		16			7
m-Iodobenzylguanidine hemisulfate		8			
6(5H)-Phenanthridinone		27	48		
1,10-Phenanthroline monohydrate				6	
Amsacrine hydrochloride	Topoisomerase II inhibition	13	37		8
Ellipticine					4
Mizoribine	Nucleotide synthesis inhibition		8		
Mitoxantrone	DNA synthesis inhibition	14			
Leflunomide	Pyrimidine synthesis inhibition	11		5	
Kenpaullone	Cyclin-dependent kinase inhibition	11		4	
Olomoucine		11			
Actinonin	Induces cell cycle arrest				8
4-Hydroxybenzhydrazide	Chelates to DNA, blocks peroxidase				6
Indirect PARP inhibitors (?): dopaminergic and adenosinergic drugs					
(±)-6-Chloro-PB hydrobromide	D1 dopamine receptor agonist (also acts as	16	25	9	7
(±)-Chloro-APB hydrobromide	an indirect PARP inhibitor; see Results)	22	25	14	14
R(+)-6-Bromo-APB hydrobromide		8	14		11
(±)-SKF 38393, N-allyl-, hydrobromide			23		
Dihydrexidine hydrochloride		8		5	
Fenoldopam bromide		19			
SKF 75670 hydrobromide				5	
SKF 83565 hydrobromide		18	23		10
SKF 83959 hydrobromide		42	8		12
SKF 89626		14			
S-(-)-Lisuride	D2 Dopamine receptor agonist	15			
Chlorprothixene hydrochloride	D2 dopamine receptor antagonist				8
S-(-)-Eticlopride hydrochloride			16		
(+)-Butaclamol hydrochloride	Dopamine receptor antagonist				5
(-)-Quinpirole hydrochloride	D2/D3 receptor agonist				7
BP 897	D3 dopamine receptor agonist	8			
U-99194A maleate	D3 dopamine receptor antagonist	15			
PD 168,077 maleate	D4 dopamine receptor agonist			7	
Apomorphine hydrochloride hemihydrate	Non-selective dopamine receptor agonist	23		5	7
Dihydroergocristine methanesulfonate	Dopamine receptor agonist,	17			
Mesulergine hydrochloride	serotonin receptor antagonist		14		
Chlorpromazine hydrochloride	Dopamine receptor antagonist	26		4	8
8-Cyclopentyl-1,3-dimethylxanthine	Adenosine A1 receptor antagonist	16			
(S)-ENBA	Adenosine A1 receptor agonist			6	6
Dipyridamole	Adenosine transport inhibitor			6	4

Table I. Continued.

Name	Action	Mean viability increase (%)			
		3 h		24 h	
		ST	PRE	ST	PRE
Anti-inflammatory drugs, antioxidants					
Hydrocortisone	Steroids			11	7
Hydrocortisone 21-hemisuccinate sodium				9	
Beclomethasone					3
Betamethasone				15	
Corticosterone					3
Etodolac	COX-inhibitors				4
Ketorolac tris salt				4	
Meclofenamic acid sodium				6	
N-(4-acetamidophenyl)indomethacinamide					6
AA-861	5-lipoxygenase inhibitor			7	
Caffeic acid phenethyl ester	NFκB inhibitor	20		4	9
Minocycline hydrochloride	Membrane protease inhibitor, inhibits cell proliferation			6	6
Luteolin	Antioxidant and radical scavenger		10		
(±)-α-Lipoic Acid	Coenzyme pyruvate dehydrogenase				13
2,3-Dimethoxy-1,4-naphthoquinone	Redox cycling agent	12		5	
ß-Lapachone	Anticancer agent, apoptosis inducer			5	
Aminoguanidine hemisulfate	NOS inhibitor				6
N-Oleoylethanolamine	Sphingolipid signaling inhibitor				5
Muscle relaxation, cholinergic drugs					
Vincristine sulfate	Microtubule assembly inhibitor	14			
ML-7	Myosin light chain kinase inhibitor	7			
(-)-Eseroline fumarate	Cholinesterase inhibitor	13	23	5	5
(-)-Scopolamine hydrobromide	Muscarinic acetylcholine receptor antagonist	11			
(-)-Scopolamine methyl nitrate		10			
(-)-Nicotine hydrogen tartrate salt	Nicotinic acetylcholine receptor agonist		12	3	7
1,1-Dimethyl-4-phenyl-piperazinium iodide			23		
Carbachol	Acetylcholine receptor agonist		3		
Carisoprodol	Skeletal muscle relaxant	21			
CB34	Peripheral benzodiazepine receptor agonist		20	4	7
Oxiracetam	Nootropic agent, muscle relaxation (?)		29		
Centrophenoxine hydrochloride	Cholinergic action (?)			5	
Adrenergic drugs, imidazoline receptor interfering agents					
Phenoxybenzamine hydrochloride	α1 adrenoceptor antagonists		25		
Moxisylyte hydrochloride				4	
A-315456		12			
Yohimbine hydrochloride	α2 adrenoceptor blockers				15
Imiloxan hydrochloride			14		
Pindolol	ß adrenoceptor blocker			4	
MHPG piperazine	Norepinephrine metabolite	10			

Continued.

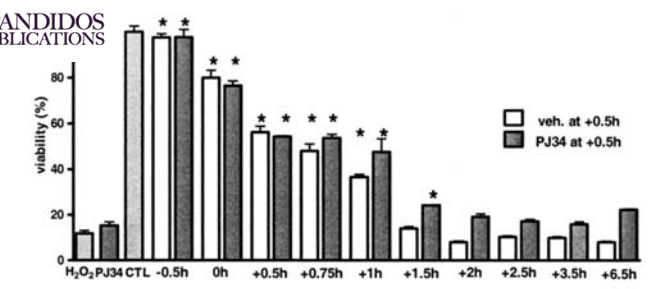
Name	Action	Mean viability increase (%)			
		3 h		24 h	
		ST	PRE	ST	PRE
Adrenergic drugs, imidazoline receptor interfering agents (continued)					
3,5-Dinitrocatechol	COMT inhibitor		14		
Quinacrine dihydrochloride	MAO inhibitor			6	
Idazoxan hydrochloride	Imidazoline I2 agonist/I1 antagonist, α adrenoceptor antagonist		16		
BU224 hydrochloride	Imidazoline I2 antagonist			11	
Ion channel blockers					
Nicardipine hydrochloride	Calcium channel blockers	8			
Nitrendipine				4	
Cinnarizine		21	25		
Diltiazem hydrochloride					10
Benzamil hydrochloride	Blocker of Na^+/H^+ and Na^+/Ca^{2+} channels	6	18		
Ouabain	Na^+-K^+ ATPases blocker				4
2,3-Butanedione monoxime	ATP-sensitive K^+ channel blocker	14			5
Amiloride hydrochloride	Na^+ channel blocker			10	
cAMP/PKA activation					
Forskolin	Adenylate cyclase activator		6	12	10
Ro 20-1724	Phosphodiesterase inhibitors			14	
8-Methoxymethyl-3-isobutyl-1-methylxanthine				6	
T-0156					11
T-1032			22		6
H-9 dihydrochloride	Inhibitors of cAMP-dependent protein kinase	12		6	
Sp-cAMPS triethylamine					8
Rp-cAMPS triethylamine					4
H-8 dihydrochloride					6
Phosphorylation inhibitors, tyrosine kinase inhibitors					
Tyrphostin AG 528	Tyrosine kinase inhibitor	18			
U0126	MEK inhibitor				8
Rottlerin	PKC and CaM kinase III inhibitor				7
Piceatannol	Non-receptor kinase Syk and Lck inhibitor		26		
Tyrphostin AG 112	Protein tyrosine kinase inhibitor				10
Tyrphostin AG 835		16			
Opioids					
Naltrindole hydrochloride	δ opioid receptor antagonist		14		
Naltriben methanesulfonate			27		
Naloxone benzoylhydrazone	κ opioid receptor antagonist	9			
nor-Binaltorphimine dihydrochloride				7	
Naloxone hydrochloride	Opioid receptor antagonist	8			

Table I. Continued.

Name	Action	Mean viability increase (%)			
		3 h		24 h	
		ST	PRE	ST	PRE
GABAergic drugs, anticonvulsive drugs					
(+)-Bicuculline	GABA-A receptor antagonist	10			
Isoguvacine hydrochloride	GABA-A receptor agonist	10			
(±)-Baclofen	GABA-B agonist, muscle relaxant				5
CGP-7930	GABA-B modulator				7
NCS-356	GABA agonist				6
N-(4-Amino-2-chlorophenyl)phthalimide	Anticonvulsive drugs		31		
Valproic acid sodium		11			
Serotoninergic drugs					
N,N-Dipropyl-5-carboxamidotryptamine maleate	5-HT1A serotonin receptor agonist			7	
Methiothepin mesylate	5-HT1 serotonin receptor antagonist				6
SB 204070 hydrochloride	5-HT4 serotonin receptor antagonist			6	
Fluoxetine hydrochloride	Selective serotonin reuptake inhibitor		7		
Histaminergic drugs					
Ketotifen fumarate	H1 Histamine receptor antagonist			5	
Terfenadine			18		
3-(1H-Imidazol-4-yl)propyl di(p-fluorophenyl) methyl ether hydrochloride	H3 Histamine receptor antagonist				4
4-Imidazolemethanol hydrochloride	Histonol dehydrogenase inhibitor		17		
NMDA glutamate receptor interfering agents					
Pentamidine isethionate	NMDA glutamate receptor antagonist		33		
Spermidine trihydrochloride	NMDA modulator			6	
DL-threo-ß-hydroxyaspartic acid	Glutamate transport inhibitor	12			
Calcimycin	Potentiates NMDA signaling		23		
Purinergic					
α,ß-Methylene adenosine 5'-triphosphate dilithium	P2 receptor agonist				9
NF 023	P2X1 receptor antagonist			5	
Others: tachykinin and capsaicin analogues					
WIN 62,577	NK1 tachykinin receptor antagonist				11
Capsazepine	Vanilloid receptor antagonists			7	
N-Vanillylnonanamide					8

[a]Drugs that exerted a cytoprotective effect in the standard (ST) or pretreatment (PRE) assays, either in 3 or 24 h, are shown with their likely mode of action. In case of cytoprotective action, normalized mean increases in viability are shown as percent values.

Chloro-APB and Bromo-APB hydrobromide are potent cytoprotective compounds. Treating the cells with chloro-APB hydrobromide 30 min after the H_2O_2 challenge that resulted in 70% cell death dose-dependently increased cell survival at 3 h. Survival exceeded 70% in the case of 30 μM chloro-APB hydrobromide (Fig. 8a). The level of cell survival seen with



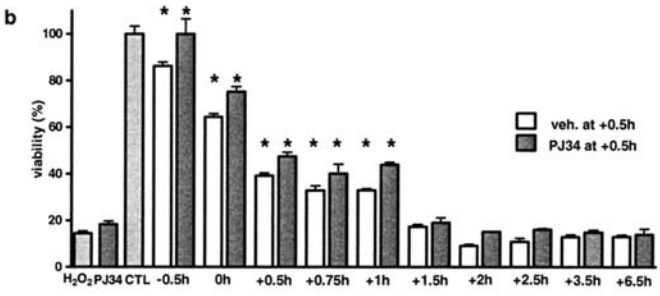
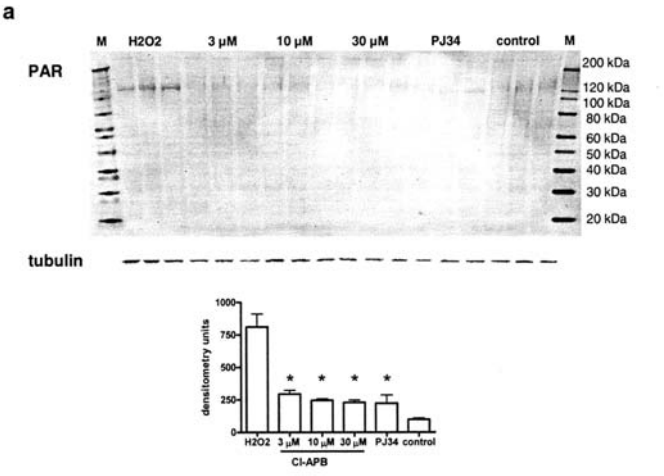
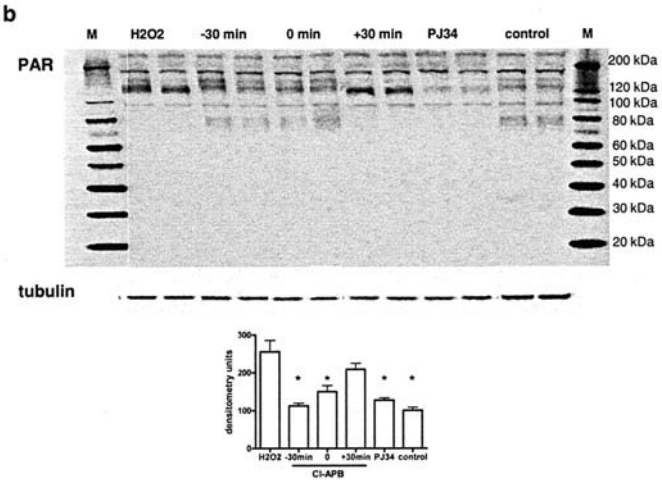


Figure 9. The cytoprotective effect of APB hydrobromide, when applied at various time points. Cardiomyocytes were challenged with H_2O_2 (1000 μM), 30 min later vehicle or 3 μM PJ34 was added, and cell viability was measured 24 h later by MTT assay. Chloro- (a) or bromo-APB hydrobromide (b) was added at 10 μM at the indicated time points. Time points are relative to the application of H_2O_2 and are shown in hours (h). Viability values are expressed as percent values of vehicle-treated cells (CTL, control); average ± SEM values are shown. *p<0.05 compared to the H_2O_2 group.





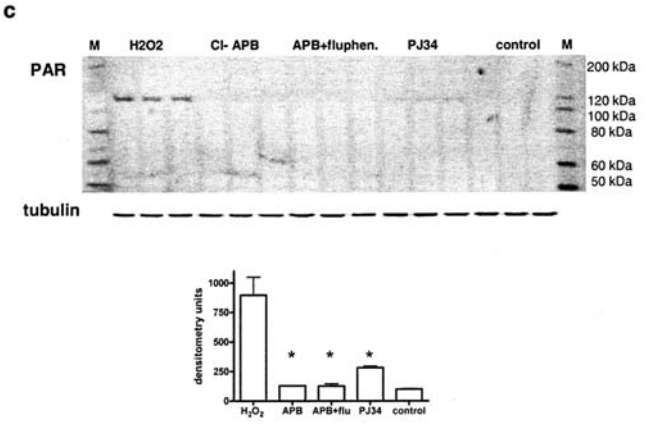


Figure 10. Cl-APB hydrobromide inhibits cellular PARP activation. Cardiomyoblasts were treated with H_2O_2 (500 μM) for 60 min, and PARP activity was measured by Western blotting for PAR. Cells were pretreated with Cl-APB hydrobromide for 30 min before the H_2O_2 challenge at the indicated concentrations (a), or received Cl-APB-hydrobromide 30 min prior to (-30 min), simultaneously with (0 min) or 30 min after (+30 min) the application of H_2O_2 (b), or Cl-APB hydrobromide was applied alone and in combination with receptor antagonist fluphenazine (APB+fluphen.) (c). The H_2O_2 group received H_2O_2 treatment only; the PJ34 group received H_2O_2 and PJ34, and the controls received vehicle only. Representative blots and densitometric analysis results are shown. PAR signals were normalized to tubulin signal and are shown as the mean ± SEM. *p<0.05 compared to the H_2O_2 group.

30 μM chloro-APB hydrobromide was comparable to the effect of the potent PARP inhibitor PJ34. However, the cytoprotective effect of APB hydrobromide was more pronounced at 24 h than that of the PARP inhibitor reference compound. Bromo-APB hydrobromide significantly reduced the H_2O_2-induced cell death at concentrations >3 μM. When H_2O_2 induced 75% reduction in viable cell count, 30 μM bromo-APB hydrobromide resulted in >40% cell survival, which was significantly higher than what was achieved by PJ34 (Fig. 8b). Combined treatment with 3 μM PJ34 and increasing concentrations of bromo-APB hydrobromide resulted in a dose-dependent increase in cell survival. The combination of APB hydrobromide and PARP inhibitor PJ34 reduced cell loss compared to the single APB treatment at all concentrations of bromo-APB hydrobromide (Fig. 8c). Bromo-APB hydrobromide (30 μM) increased cell survival from 25-40%, and the combination with 3 μM PJ34 further increased cell survival to 45%. The chloro-APB hydrobromide-induced improvement in cell viability increase was paralleled with significantly reduced LDH release, i.e. lower LDH activity was detected in the cell culture supernatant (Fig. 8d). Combined treatment with PJ34 and APB hydrobromide further reduced the LDH release, reaching nearly baseline levels when 3 μM PJ34 and 30 μM APB hydrobromide was applied (Fig. 8e).

To study the effective time-frame when APB hydrobromide reduces cytotoxicity, cells were either pretreated with the compound, applied simultaneously with or after the H_2O_2 challenge at various time points. A significant increase

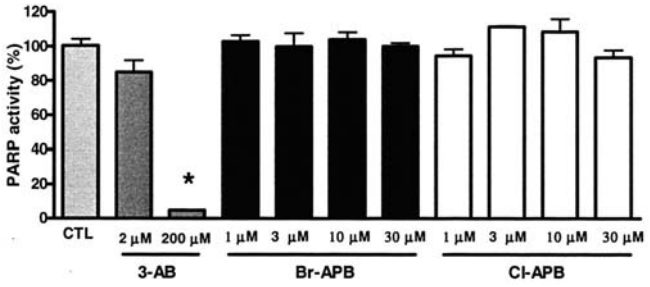


Figure 11. Direct PARP assay. Purified PARP enzyme was pre-incubated with PARP inhibitor 3-aminobenzamide (3-AB), bromo-APB hydrobromide or chloro-APB hydrobromide at the indicated concentrations. PARP was activated with sheared DNA and incubated with labeled NAD substrate. PARP activity was determined by the measurement of incorporated labeled ADP-ribose residues, and was expressed as percent values of maximum PARP activity (vehicle treatment, CTL). Mean ± SEM values are shown.

was detected in cell survival when peroxide was used prior to or concurrently with the oxidative injury. APB treatment following the oxidative challenge also significantly reduced cell loss at 24 h, when it was applied within the first hour (Fig. 9). The highest cell survival was detected when APB-hydrobromide was added 30 min prior to the H_2O_2 challenge, and cell survival with later treatments decreased in a time-dependent manner. There was no difference in this pattern between bromo- and chloro-APB hydrobromide. Pretreatment with either APB-hydrobromide molecules at 10 μM nearly completely restored cell viability when peroxide-induced injury resulted in 80% cytotoxicity.

APB hydrobromide is an indirect inhibitor of cellular PARP activity. The cytoprotection of APB hydrobromide was comparable to the protection afforded by the PARP inhibitor PJ34 in the H_2O_2-induced cell death model and only moderate improvement was detected when APB molecules were combined with PJ34. To test whether PARP inhibition plays a role in the cytoprotective effect of APB molecules, confluent cultures of H9c2 cardiomyocytes were treated with H_2O_2 to induce PARP activation, and poly(ADP-ribose) (PAR) was detected by Western blotting. H_2O_2 induced protein poly(ADP-ribosylation) that could be detected at 120 kDa, which corresponds to the auto-ribosylation of PARP. APB hydrobromide decreased poly(ADP-ribosylation) at 120 kDa, similar to PJ34, nearly to baseline, at 3-30 μM concentrations (Fig. 10a). The D1 receptor antagonist fluphenazine had no impact on the PARP inhibitory effect of APB hydrobromide (Fig. 10b), consistent with the notion that the cytoprotective effect of these compounds is due to a chemical mechanism other than interaction with the cellular D1 receptors. This was predicted by the facts that the cardiomyocytes used in the current assay are not known to express functional D1 receptors and the rank order of potency between the currently used D1 compounds did not mirror their rank order of potency (31,32) as D1 receptor agonists. Indeed, pretreatment of the cells with fluphenazine at 10-30 μM (or with chlorpromazine at 3-30 μM) did not attenuate the cytoprotective effects of these D1 receptor ligands (data not shown). It is noteworthy that the marked cytoprotection that APB hydrobromide provided at 24 h differed from that of the PARP inhibitor PJ34, which was only marginally active by this time.

The PARP inhibitory function of APB hydrobromide was also examined in a cell-free system. In a direct PARP assay, when PARP enzyme is activated by sheared DNA, the PARP inhibitor 3-aminobenzamide reduced poly(ADP-ribosylation) in a concentration dependent manner, whereas no significant reduction in PARP activity was detected with either chloro- or bromo-APB hydrobromide (Fig. 11).

In conclusion, the findings reported here demonstrate that cytoprotective agents, including novel indirect inhibitors of cellular PARP activation can be identified with a cell-based high-throughput screening method, chloro-APB hydrobromide being one such compound. Cell-based high-throughput screening (as opposed to the traditional process of drug target identification, followed by high-throughput screening of the target enzyme) has recently been proposed by several experts as the method of choice to identify novel lead compounds influencing complex cellular responses where a significant degree of redundancy is present in the system (33-38). The results of the current study lend further support to the validity of this approach. The current method will be applicable for cell-based high-throughput screening of various additional compound libraries, in order to identify bioactive lead molecules and/or to find bioactive functional synergists or functional antagonists of compounds that influence complex cellular responses.

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