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Poly(ADP-ribose) glycohydrolase as a target for neuroprotective intervention: assessment of currently available pharmacological tools

Jeppe Falsig[a,b,*], Søren Hofman Christiansen[a], Sascha Feuerhahn[c], Alexander Bürkle[c], Shiao Li Oei[d], Claudia Keil[d], Marcel Leist[a]

[a]*H. Lundbeck A/S, 2500 Valby, Denmark*
[b]*Institut für Neuropathologie, Universität Zürich, CH-8091 Zürich, Switzerland*
[c]*Laboratory of Molecular Toxicology, Department of Biology, University of Konstanz, D-78457 Konstanz, Germany*
[d]*Institute for Biochemistry, Freie University Berlin, D-14195 Berlin, Germany*

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Abstract

Poly(ADP-ribose) glycohydrolase (PARG) is being considered as a therapeutic target for the prevention of neurodegeneration. Here, we assessed the pharmacological tools available for target validation. The tannic acid derivative gallotannin inhibited PARG in a cell-free assay but had no detectable effect on PARG function in intact cells. Its cytoprotective actions were associated rather with the radical-scavenging potential of the compound. In astrocytes exposed to high concentrations of the nonoxidative DNA-damaging agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), Poly(ADP-ribose) polymerase (PARP) inhibitors were fully protective, while gallotannin enhanced the damage. The compound *N*-bis-(3-phenyl-propyl)9-oxo-fluorene-2,7-diamide (GPI 16552), considered a potentially specific PARG inhibitor, had no effect in the different astrocyte death models compared with PARP inhibitors. In an in vitro PARG activity assay, the maximal inhibition that could be achieved with GPI 16552 was only 40% at a drug concentration of 80 μM. We conclude that neither GPI 16552 nor gallotannin are suitable for the evaluation of PARG in cellular death models, and that previous conclusions drawn from the use of these compounds should be interpreted with caution.

Keywords: PARG; PARP; Gallotannin; GPI 16552; Astrocyte; Cell death

1. Introduction

Poly(ADP-ribosyl)ation and the enzymes involved have attracted large attention as targets for pharmacological intervention under conditions of tissue injury. Poly(ADP-ribose) polymerase-1 (PARP-1, EC 2.4.2.30) is the major nuclear enzyme with poly(ADP-ribosyl)ating activity. The substrate of this enzymatic reaction is β-NAD^+, which yields the ADP-ribosyl moiety used for the transfer reaction, and nicotinamide, which is released. The catalytic activity of PARP-1 depends on the presence of DNA single- or double-strand breaks, which are sensed via two zinc fingers in a specific domain of the enzyme. In the activated state, PARP-1 adds chains of poly(ADP-ribose; PAR) consisting of up to 200 ADP-ribosyl units to various nuclear proteins, including histones and PARP-1 itself (Satoh et al., 1994; Pieper et al., 1999). The covalently attached highly negatively charged poly(ADP-ribose) affects protein function and leads to chromatin decondensation, making the DNA strands accessible to DNA repair enzymes (de Murcia et al., 1988). In replicating cells and tissues under mild to moderate genotoxic stress, PARP-1 activity is a survival factor and a guardian of the genome (Ishizuka et al., 2003; for review: Bürkle, 2001). In stark contrast, PARP-$1^{-/-}$ mice show resistance to cerebral or myocardial ischemia, 1-methyl-4-

* Corresponding author. Institut für Neuropathologie, Universität Zürich, Schmelzbergstrasse 12, CH-8091 Zürich, Switzerland. Tel.: +41 1 255 3931; fax: +41 1 255 4402.
E-mail address: jeppefalsig.pedersen@usz.ch (J. Falsig).

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phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson syndrome, streptozotocin-induced diabetes, as well as to many forms of inflammation in animal models (Ying et al., 2001; Ha et al., 2002). This suggests a role for PARP-1 in neurodegeneration and tissue destruction. Apparently, excessive activation of PARP-1 under such pathophysiological conditions results in cellular depletion of the NAD^+ pool with important implications for energy metabolism (Pieper et al., 1999). Moreover, cellular ATP depletion can occur as a secondary consequence, because NAD^+ resynthesis is an energy and adenine nucleotide-demanding process.

Poly(ADP-ribose) glycohydrolase (PARG) negatively regulates the cellular amount of PAR by degrading the PAR chains synthesised by PARP-1 and a number of additional PARP isoforms (Chiarugi, 2002). PARG is an endoexoglycosidase, with a low cellular expression but with a high specific activity, as shown by the fast turnover of poly(ADP-ribose), which has a half life of less than 1 min under conditions of DNA breakage (Alvarez-Gonzalez and Althaus, 1989). Little information is available on the normal cellular function of PARG, (for review, see Davidovic et al., 2001), but it has been suggested that PARG is involved in PARP-1-dependent cell death (Ying et al., 2001). During excessive PARP activation, the simultaneous activity of PARG would result in a vicious NAD^+-consuming cycle of synthesis and degradation of PAR. This would result in a complete degradation of NAD^+ and of ATP, ultimately converting it to nicotinamide and free monomeric ADP-ribose.

PARP inhibitors like 1,5 dihydroxyisoquinoline and 3-aminobenzamide have been shown to be protective in many models of cell death associated with PARP-1 overactivation (Purnell and Whish, 1980; Banasik et al., 1992; Szabo and Dawson, 1998), and recently, four groups have claimed that PARG inhibitors also are protective in the very same models (Ying and Swanson, 2000; Ying et al., 2001; Hwang et al., 2002; Kim and Koh, 2002; Lu et al., 2003). It was suggested that the mechanisms of action was PARG inhibition preventing the NAD^+-consuming vicious cycle, and causing inhibition of PARP-1 due to excessive auto-ADP-ribosylation, which blocks the enzymatic activity of PARP-1 (Zahradka and Ebisuzaki, 1982). These data are mainly based on the use of gallotannin as a putatively selective PARG inhibitor.

In cell-free assays, gallotannin, a complex mixture of tannins purified from oak gall, has been shown to inhibit PARG (Tsai et al., 1992; Aoki et al., 1993), and the compound also showed protection in cell culture models of oxidative stress-induced cell death (Ying and Swanson, 2000; Ying et al., 2001). Another small molecule with putative PARG-inhibitory capacity, *N*-bis-(3-phenyl-propyl)9-oxo-fluorene-2,7-diamide (GPI-16552), was reported to significantly reduce the infarct volume in a middle cerebral artery occlusion model of cerebral ischemia (Lu et al., 2003). Furthermore, it was postulated that PARG might affect inflammation by inhibition of inducible nitric oxide synthase (iNOS), (Chiesi and Schwaller, 1995). Here, we used astrocyte models to examine putative PARG inhibitors. We used three PARP-1-dependent cell death models plus one inflammation model in primary astrocytes to reinvestigate the effect of GPI-16552 and gallotannin.

2. Materials and methods

2.1. Materials

Complete cytokine mix (CCM) contained 10 ng/ml murine tumor necrosis factor-alpha (TNF-α), 10 ng/ml murine interleukin-1β (Sigma-Aldrich, Copenhagen, Denmark), and 5 U/ml recombinant murine interferon gamma (IFN-γ; R&D Systems, Abingdon, UK). Other reagents were *S*-nitroso-*N*-acetylpenicillamine (SNAP) and 3-morpholinosydnonimine (SIN-1) purchased from Bie and Berntsen (Rødovre, Denmark). Basic laboratory chemicals and inhibitors were purchased from Sigma, unless stated otherwise. GPI-16552 [*N*-bis-(3-phenyl-propyl)9-oxo-fluorene-2,7-diamide] was synthesised as described (WO 02057211; Li et al., 2002) with the following minor modifications: 9-fluorenone-2,7-diacyl chloride was prepared by refluxing a mixture of thionyl chloride and 9-fluorenone-2,7-dicarboxylic acid in N,N-dimethylformamide for 10 h. The crude product underwent reaction with 3-phenylpropyl amine in a DMF–pyridine mixture to yield 2,7-Bis[*N*-3(phenyl-propyl)carbamoyl-9-oxo-9H-fluorene.

2.2. Primary astrocyte culture

Pregnant C57bl/6jbom mice were purchased from Harlan (Horst, The Netherlands). All experimental procedures were carried out in accordance with the directives of the Danish National Committee on Animal Research Ethics and the European Communities Council Directive #86/609 for care of laboratory animals.

Primary cortical astrocytes were prepared as described (Falsig et al., 2004) from 1- to 2-day-old mice according to a slightly modified version of a protocol by David E. Weinstein (Weinstein, 1997). In brief, brains from six pups were removed and kept on ice in phosphate-buffered saline (PBS) containing 2 g/l glucose and 0.001% (w/v) phenol red, pH 7.4 (PBS-G). The cortices were dissected, and the hippocampi and meninges were carefully removed before digestion in PBS-G containing 10 mg/ml trypsin TRL (Worthington, Lakewood, USA), 1 mg/ml DNase (Worthington), and 5 mg/ml $MgSO_4$ for 3 min at 37 °C. Tissue was triturated in PBS-G with 0.5 mg/ml DNase. Cells were carefully layered over a 30% Percoll solution (Amersham Pharmacia Biotech, Hørsholm, Denmark) in PBS-G and centrifuged at 150×*g* for 10 min. Astrocytes were recovered from the interface, washed once with 15 ml PBS-G (100×*g* for 5 min), and resuspended in Dulbecco's Modified Eagle's

Medium (DMEM; high glucose), 10% foetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. This medium was used for culturing both primary cells and cell lines. All medium ingredients were from Invitrogen (Taastrup, Denmark). Cells were seeded at a density of 10,000 cells/cm^2 in 185-cm^2 flasks (NUNC, Roskilde, Denmark) and placed at 37 °C, 5% CO_2, 95% relative humidity. Medium was changed after 3 days and subsequently twice a week. Cells were trypsinised and reseeded after 14 days in primary culture at a density of 30,000 cells/cm^2 and were always used for experiments 6–8 days after replating.

2.3. Cell-free PAR-assay

^{32}P-PAR was synthesized and purified in vitro using purified PARP as described earlier (Griesenbeck et al., 1997). After deproteinization and purification, isolated ^{32}P-PAR consisted of long and branched polymers with chain length far above 30 ADP-ribose units. PARG activities were determined as described previously (Keil et al., 2004). In brief, 100 ng recombinant PARG (aa 378–976) was incubated in 30 μl phosphate buffer (50 mM potassium phosphate pH 7.2, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10 μg/ml bovine serum albumin) at 37 °C for 20 min. Reactions were stopped by precipitation with acetone. Precipitated nucleotides were redissolved in Tris/EDTA (TE)-buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Samples containing equal amounts of radioactivity were applied to cellulose-coated plates (Macherey-Nagel, Düren, Germany). Thin-layer chromatography was performed using the solvent system 0.3 M LiCl'1 M acetic acid. After separation, dried cellulose plates were subjected to autoradiography or quantified using a Phospho imager.

2.4. Cell-based PAR-assay

This was performed as described previously (Bürkle et al., 1993), with minor modifications. Briefly, HeLa cells were cultured in standard cell culture medium and replated for experiments at a density of 3–5×10^4 cells per 12 mm cover slip (placed in 12-well plate). After 3 days, the medium was replaced with DMEM without FCS, and the cells were pretreated with gallotannin (30 μM) or GPI-16552 (14 μM) for 1.5 h (all experiments were vehicle-controlled). After this time, *N*-Methyl-*Ń*-nitro-*N*-nitrosoguanidine (MNNG; in case of GPI-16552) or H_2O_2 was added to the wells to yield final concentrations of 50 μM and 1 mM, respectively. After 10, 20, or 30 min of incubation at 37 °C, the reaction was stopped by transfer of the cover slip to a 12-well plate containing 10% trichloroacetic acid (TCA). Each cover slip was washed for 5 min consecutively in 70%, 90%, and 100% EtOH (−20 °C) on ice. After air-drying over night, the cover slips were rehydrated in PBS for 5 min. Cells were incubated with monoclonal PAR antibody 10H (Kawamitsu et al., 1984) at 5 μg/ml in PBS+0.05% Tween+5% powdered milk for 30 min at 37 °C, followed by a 4×5 min washing step in PBS. The secondary antibody [AlexaFluor488 goat anti-mouse immunoglobulin (IgG), diluted 2 μg/ml in PBS+5% powdered milk] was incubated for 20 min at 37 °C followed by 4×5 min of washing in PBS. Cell nuclei were counterstained with Hoechst 33342 (0.25 μg/ml in PBS) and mounted on glass slides using Aquapolymount (Polysciences, Eppelheim, Germany). Pictures were taken using Zeiss Axiovert S100TV fluorescence microscope equipped with a Hamamatsu Orca camera, through a 63× oil-immersion lens. Pictures were recorded at an exposure time of 2 s, except for the 10-min time points, corresponding to the peak of PAR accumulation, where exposure time was set at 0.5 s. Imaging software used was Aquacosmos version 2.0 from Hamamatsu (Hamamatsu Photonics GmbH, Herrsching, Germany).

2.5. Standard cell incubation scheme

2.5.1. Cell death model

Cells were preincubated with inhibitors for 30 min in DMEM (high glucose), 2% FCS, 1% P/S before addition of damaging compounds. After 24 h, cell death was measured by lactate dehydrogenase (LDH) release assay. Careful titration of H_2O_2, MNNG, and SIN-1 was performed, and the following concentrations were chosen for their ability to induce 80–100% cell death: H_2O_2 300 μM, MNNG 100 μM, and SIN-1 5 mM. Alternative incubation schemes were also tried. Cells were preincubated with inhibitors for 60 min in medium; cells were washed and incubated for 60 min with MNNG with or without inhibitors present. Cells were washed and inhibitors were readded to all wells, and after 24 h, MTT reduction was measured. To exclude any interference by serum, gallotannin was also tested in a balanced salt solution (BSS; 3.1 mM KCl, 134 mM NaCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.25 mM KH_2PO_4, 15.7 mM $NaHCO_3$, 2 mM glucose, pH 7.2). All inhibitor stocks were diluted in BSS, and the pH was adjusted to 7.2. Cells were preincubated for 30 min with inhibitors before the addition of MNNG. After 60 min, MNNG was washed away, and the cells were incubated in Eagle's MEM plus 0.1% bovine serum albumin for 23 h.

2.5.2. CCM model

Cells were preincubated with inhibitors for 30 min before adding a mixture of cytokines (CCM) consisting of TNF-α (10 ng/ml), interleukin-1β (10 ng/ml), and IFN-γ (5 U/ml). After 24 h, nitric oxide (NO) production was quantified by measuring the accumulation of the NO metabolite nitrite in the astrocyte-conditioned medium.

2.5.3. Cell-free NO donor assay

NO donors freshly dissolved in dimethyl sulfoxide (SNAP) or medium (GSNO) were diluted in DMEM (high glucose), 2% FCS, 1% P/S. Under these conditions, NO

donors will decompose. After 16 h, accumulated nitrite was measured. All experiments were vehicle-controlled.

2.6. Nitrite measurement

Nitrite was measured by the use of the Griess reagent. In brief, 50 μl supernatant or $NaNO_2$ standards were mixed with 25 μl *N*-(1-naphtyl) ethylendamine (0.1% in H_2O) and 25 μl sulfanilamide (1% in 1.2N HCl) in a 96-well plate. After 3 min, samples were read at (570–690 nm).

2.7. Measurement of cell death

LDH release was measured using a cytotoxicity detection kit (Roche) as follows: cell culture supernatant was sampled before lysis of the cells in 0.1% Triton X100 in medium. An equal amount of cell lysate and supernatant was incubated with the reagent mixture, and after 15 min of incubation at room temperature, the formazan product was measured spectrophotometrically at 492–690 nm. Data are displayed as the percentage of LDH activity in the supernatant compared to total LDH activity in the well. A positive control routinely yielded an LDH release of 95%. This value was set to 100% release, and all samples were normalised to the positive control.

Cell viability was also assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], a yellow tetrazolium salt that is reduced to a blue formazan product within functional mitochondria of living cells. Cells were incubated with MTT (0.5 mg/ml) dissolved in cell culture medium for 2 h. After the incubation period, medium was removed, and the formed Formazan crystals were lysed in 100 μl lysis buffer (isopropanol: formic acid, 95:5) under vigorous shaking. Absorption was measured at 570–690 nm on the Multiskan plate reader.

2.8. Western analysis

The protein levels of iNOS and beta-actin were assessed using Western blot analysis. Astrocytes were washed once in PBS before lysis in ice-cold buffer containing 1% NP-40, 20 mM Tris–HCl pH 8.0, 137 mM NaCl, 10% glycerol, 4 mM iodo acetamide, 10 mM NaF, 1 mM 4-(2-aminoethyl)-benzene sulifonyl fluoride hydrochloric-acid (AEBSF), 1 mM Na_3VO_4, and "Complete mini" protease inhibitor mix from Roche (1 pill/10 ml buffer). The lysate was transferred to a microfuge tube and incubated for 15 min on ice before cellular debris was spun down for 10 min at 10,000×*g*, 4 °C. The supernatants were transferred to a fresh tube and stored at −80 °C. The protein concentration was determined by the bichionic acid method (BCA) using a commercial kit (Pierce). The NuPAGE-kit [4-12% Bis–Tris gel run with 4-morpholinepropanesulfonic acid (MOPS) buffer under reducing conditions; Invitrogen] was used for electrophoresis according to the manufacturer's instruction. Gels were run for approximately 1 h at 200 V before blotting proteins onto an activated polyvinylidene fluoride (PVDF)-membrane (Immobilon P, Millipore, Glostrup, Denmark) using wet-transfer blot module XCell2 (Invitrogen). The membranes were cut in two halves at roughly 50 kDa, blocked with 5% milk in TBST (2.42 g/l Tris–HCl, 8 g/l NaCl, and 0.1% Tween-20 pH 7.6), washed with TBST, and incubated with primary antibody dissolved in 5% milk in TBST overnight at 4 °C. Blots were washed and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature and developed using ECL or ECL+ (Amersham Pharmacia Biotech, Hillerød, Denmark). Primary antibodies used were rabbit polyclonal antimouse iNOS (1:2000; Alexis). Mouse anti-β-Actin clone AC-15 (Sigma-Aldrich; 1:5000) was used to control for equal loading of lanes. Secondary antibodies used were rabbit antimouse 1:2000 and goat antirabbit 1:1000 (DAKO, Glostrup, Denmark).

2.9. Caco-2 assay

Caco-2 cells (an intestinal epithelial cell-line; ATCC Number HTB-37) were seeded in culture inserts, placed in 12-well plates, and differentiated according to Chantret et al., 1988. Before the experiment was started, the trans-epithelial electrical resistance was measured to ensure a tight seal between the cells separating the two chambers. This measurement was repeated after the experiment was completed to ensure the continued integrity of the cell layer. In all wells containing cells, the transepithelial electrical resistance was above 400 Ω both before and after the experiment. Twenty-five micromolar gallotannin was added either to the insert chamber or to the reservoir chamber in a final volume of 500 and 1000 μl, respectively. After 8 or 24 h, the conditioned medium in the top and the bottom chamber was tested for the ability to rescue astrocytes from an exposure to 300 μM H_2O_2 as described previously (standard cell incubation scheme). As a positive control, either fresh gallotannin or gallotannin added to inserts containing no Caco-2 cells were used.

3. Results

3.1. Models of oxidative and nonoxidative poly(ADP ribose)-mediated cytotoxicity

Three different PARP-1-dependent cell death models were set up in primary cultures of murine fetal astrocytes. DNA strand breaks were triggered either by hydrogen peroxide (through the formation of hydroxyl radicals in the Fenton reaction), by SIN-1 (producing nitric oxide and superoxide, together forming peroxynitrite), or by MNNG (an alkylating agent). All insults were carefully titrated (data not shown), and a concentration inducing near-maximal (80–100%) cell death over a 24-h period was chosen. To verify the PARP-1 dependence of all models, two PARP

inhibitors, 1,5-dihydroxyisoquinoline (DHQ) and 3-aminobenzamide, respectively, were tested. Both inhibitors worked within the range of concentrations published previously by others (Shah et al., 1996; Fig. 1A,B). The apparently lower potency in the SIN-1 model was most probably due to a higher intensity of the insult.

3.2. Protection by gallotannin correlates with its antioxidant properties

Having established our models as being PARP-1-dependent, we next tested two putative PARG inhibitors. As expected, gallotannin blocked almost completely all cell death in the H_2O_2 and SIN-1 models. gallotannin enhanced, however, the cell death in the MNNG model in a concentration-dependent fashion (Fig. 2A). Gallotannin was tested extensively in the MNNG model with different incubation schemes, different cell death endpoints, in the presence or absence of serum, with BSS instead of medium, or with an insult inducing only 50% death. The outcome of all these experiments was similar; that is, gallotannin did not rescue cells in the MNNG model (Fig. 3B and data not shown). GPI-16552 was also tested but no effect was seen even at concentrations as high as 140 μM (approximately 90 times the reported IC_{50} value; Lu et al., 2003; Fig. 2B). A small effect was seen on SIN-1-induced cell death at these high GPI concentrations, apparently due to the high concentration of dimethyl sulfoxide necessary to keep GPI-16552 soluble at high concentrations.

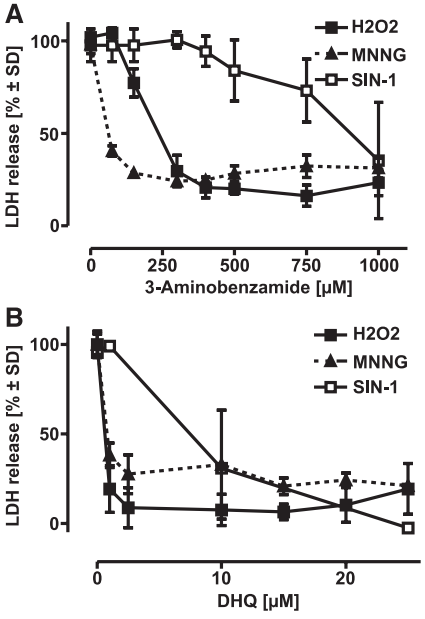

Fig. 1. Effect of PARP inhibitors in DNA damage-induced cell death models. Primary astrocytes were pretreated for 30 min with PARP inhibitors, (A) 3-aminobenzamide and (B) 1,5 isoquinolinediol. Three different cell death-inducing agents were added: (■) hydrogen peroxide (300 μM), (▲) MNNG (100 μM), or (□) SIN-1 (5 mM). LDH release was measured after 24 h. All data are displayed as means of triplicate determinations±standard deviation (S.D.).

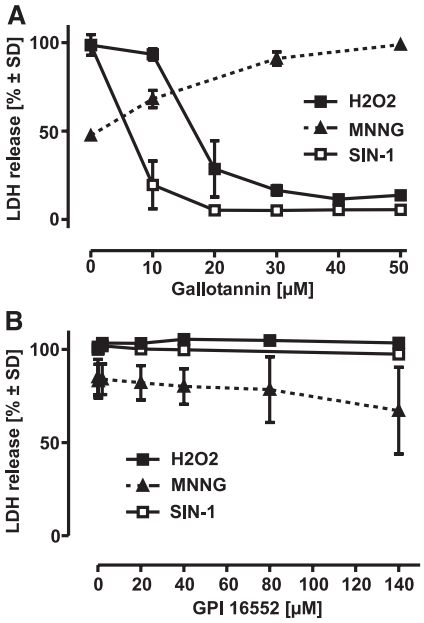

Fig. 2. Effect of PARG inhibitors in DNA damage-induced cell death models. Primary astrocytes were pretreated for 30 min with putative PARG inhibitors, (A) gallotannin or (B) GPI-16552. Three different cell death-inducing agents were added: (■) hydrogen peroxide (300 μM), (▲) MNNG (100 μM), or (□) SIN-1 (5 mM). LDH release was measured after 24 h. All data are displayed as mean LDH release±S.D.

Next we tested two other polyphenols, not reported to be PARG inhibitors, in the hydrogen peroxide model. Quercetin (five phenol groups and one resonating oxo group) and also to some extent even catechin (five phenol groups) blocked cell death in the model at concentrations similar to gallotannin, suggesting that PARG inhibition is not necessary for cytoprotection by polyphenols (Fig. 4).

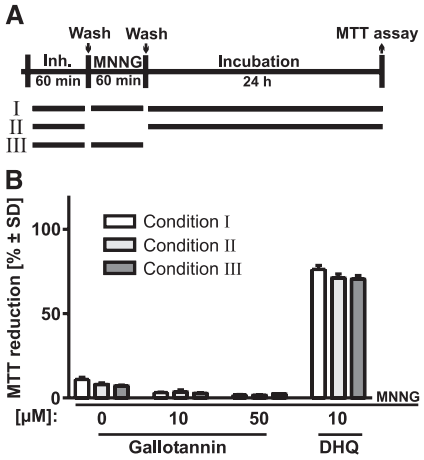

Fig. 3. PARP, but not PARG inhibitors, prevent cell death in the MNNG model independent of incubation scheme and method of cell death measurement. (A) Different incubation schemes indicated as conditions I, II, or III, respectively. (B) Cells were pretreated for 60 min with the PARG inhibitor gallotannin (10–50 μM) or the PARP inhibitor 1,5-isoquinolinediol (10 μM). After a washing step, the cells were incubated for 60 min with MNNG. MTT reduction was measured after 24 h further incubation (in condition 2 MNNG was present during this time). Data are displayed as percent MTT reduction compared to untreated controls (=100±).

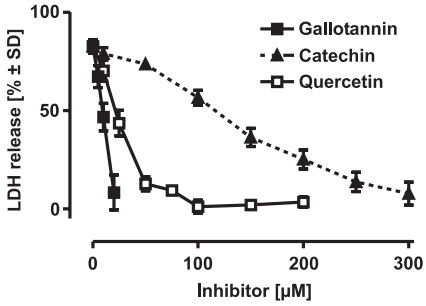

Fig. 4. Protection by nonspecific polyphenols in models of oxidative stress. Primary astrocyte cultures were pretreated for 30 min with (■) gallotannin, (▲) catechin, or (□) quercetin. Hydrogen peroxide (300 μM) was added, and after 24 h, LDH release was measured. All data are displayed as means of triplicates ±S.D.

3.3. Absence of PAR overaccumulation expected to result from PARG inhibition by gallotannin in living cells

In order to measure the drug effects on cellular PAR activity more directly, we developed an assay for direct measurement of PAR accumulation in HeLa cells treated with hydrogen peroxide together with the two putative PARG or one PARP inhibitor. Ten minutes after exposure to hydrogen peroxide, cells became strongly immunoreactive for PAR (Fig. 5A). As expected, PAR was then rapidly degraded, and apart from some faint nuclear speckles, the immunoreactivity almost completely vanished after 20 min (Fig. 5A). The experimental system was validated by use of the PARP inhibitor 3-aminobenzamide, which blocked the PAR formation by >90% (Fig. 5B). Gallotannin also reduced the PAR formation. This result would have been expected from a reactive oxygen species scavenger. A selective PARG inhibitor is expected to enhance PAR accumulation and/or delayed the time-dependent loss PAR immunostaining. However, gallotannin did not enhance the staining for PAR at any time point (Fig. 5B). GPI-16552 did not seem to have any significant effects on PAR formation (data not shown).

3.4. Inhibition of the catalytic domain of PARG by gallotannin and GPI-16552

We next examined the effect of gallotannin or GPI-16552 on the catalytic activity of PARG in a cell-free assay. The catalytic domain of human PARG (aa 378–976) was overexpressed in *E. coli* and purified to homogeneity as described before (Keil et al., 2004). Using ^{32}P-labeled PAR polymers (branched polymers with chain length far above 30 ADP-ribose units), the recombinant enzyme was able to catalyze the release of ADP-ribose efficiently. In absence of

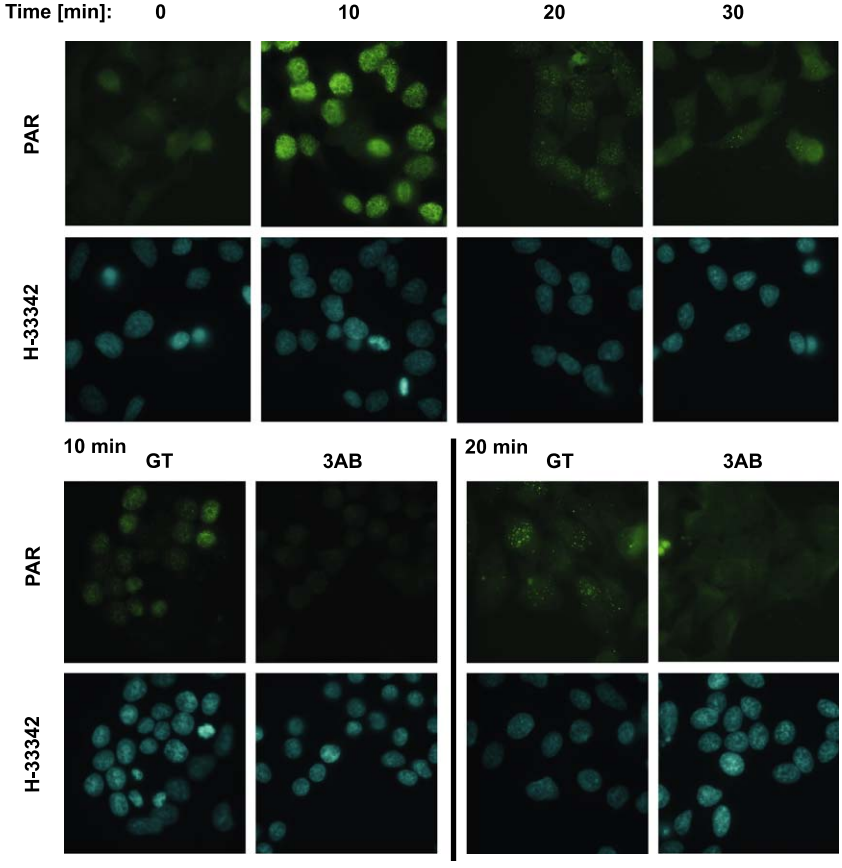

Fig. 5. Antioxidative effects of gallotannin in a cellular PAR assay. Astrocytes were pretreated with PARP or PARG inhibitors for 30 min and exposed to 1 mM H_2O_2 for 0–30 min before fixation. Cells were immunostained for PAR, and digital images were recorded as described in Materials and methods. Multiple pictures from each well were taken, and a representative picture is shown, respectively.

any inhibitor, purified PARG degraded 60% of ^{32}P-labeled polymers within 20 min of incubation (Fig. 6). Gallotannin inhibited PAR degradation almost completely at 5–10 μM suggesting that this compound is indeed a potent inhibitor of PARG in vitro as previously published by other groups (Aoki et al., 1993). GPI-16552 also inhibited PARG activity, but this compound worked in our assay only at concentrations 50 times higher than reported, and the maximum inhibition we achieved was only 40%. In the next experiment, an accumulation of PAR was analysed in vitro using PARG inhibitors as described recently (Keil et al., 2004). In brief, HeLa cell extracts were treated with nicked DNA in the presence of ^{32}P-labeled NAD$^+$ and incorporation of ^{32}P-ADP-ribose was determined. With tannin concentrations above 100 μM, a significant increase of PAR formation was obtained (Keil et al., 2004), while in the presence of GPI-16552, no obvious accumulation of PAR could be detected (data not shown). Because the influence of GPI-16552 on PARP activity has not been tested, we cannot exclude that the lack of PAR accumulation after GPI treatment is due to PARP inhibition rather the lack of PARG inhibition.

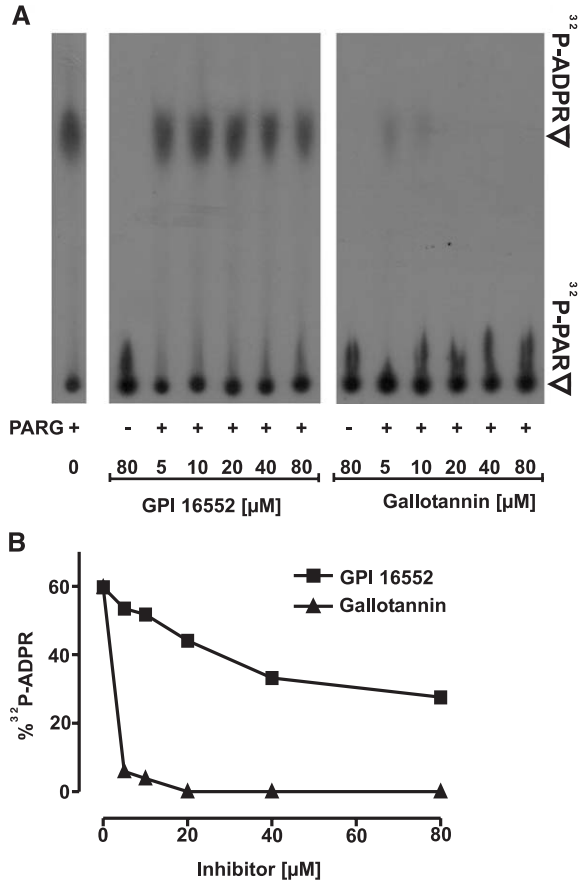

Fig. 6. Inhibition of recombinant PARG in vitro. (A) Deproteinized ^{32}P-PAR was incubated with recombinant human PARG in the presence of varying amounts of gallotannin or GPI 16552 as indicated. After 20 min, nucleotides were precipitated with acetone, separated by thin-layer chromatography, and subjected to autoradiography. Positions of PAR and ADP-ribose (ADPR) are indicated. (B) The fractions of ADPR release (in percent) were quantified using a Phospho imager.

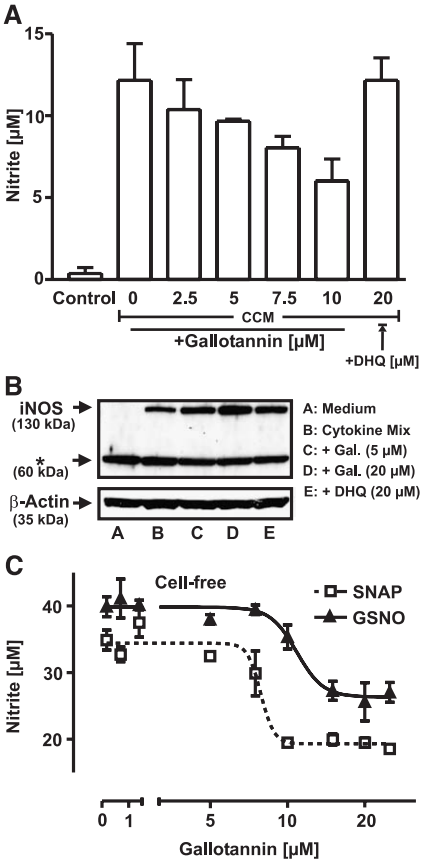

Fig. 7. Gallotannin, but not isoquinolinediol, prevents nitrite formation from nitric oxide. (A) Primary astrocyte cultures were pretreated with PARP or PARG inhibitors for 30 min before addition of a cytokine mixture (CCM) consisting of IL-1β, TNF-α, and IFN-γ. After 24 h, nitrite accumulated in the conditioned medium was quantified. All data are displayed as means of triplicates±S.D. (B) Cells were treated as in (A), and Western blot analysis of cell lysates was performed using an anti-iNOS antibody. An anti-β-actin antibody was used control equal loading. *Nonspecific band. (C) In a cell-free assay, the NO-donors, (■) SNAP (50 μM) or (▲) GSNO (50 μM), were incubated with various concentrations of gallotannin. Nitrite accumulated in the medium was determined after 16 h. All data are displayed as means of triplicates±S.D.

3.5. Scavenging of inflammatory nitric oxide by gallotannin

Due to its antioxidant properties, gallotannin might show some anti-inflammatory and cytoprotective effects in complex biological models even without entering cells and interfering with poly(ADP-ribosyl)ation. This may explain the beneficial effects of the compound completely independent of PARG inhibition. Because interference of gallotannin with NO formation in cells has already been described, we investigated more closely the underlying mode of action. First, we used a validated model of astrocyte inflammation involving the production of NO (Falsig et al., 2004). Primary astrocytes were stimulated with a complex mixture of proinflammatory cytokines (CCM) containing TNF-α, interleukin-1β, and IFN-γ. After 24 h, iNOS activity was quantified by measurement of nitrite accumulated in the cell-conditioned medium. Coad-

ministration of gallotannin caused a strong inhibition of nitrite accumulation, while the PARP-1 inhibitor DHQ had no effect (Fig. 7A). This inhibition was not due to transcriptional or translational inhibition, because gallotannin rather increased the levels of iNOS protein (Fig. 7B). We tested the hypothesis that gallotannin worked as a scavenger of NO without ever interfering with cellular processes. For this purpose, NO was generated in cell-free systems from two chemically different types of NO donors. Our data clearly showed that gallotannin blocked the accumulation of nitrite to a similar extent and with a concentration–effect relationship similar to that of the cellular system (Fig. 7C). All of the beneficial properties of gallotannin we have observed could be consistent with extracellular antioxidant effects of the compound. This prompted us to examine whether gallotannin can pass cellular membranes at all. We tested gallotannin in a standard experimental model system used to address this question, the Caco-2 intestinal epithelial cell transport assay. We found no evidence to suggest that gallotannin would penetrate the cellular monolayer (data not shown).

4. Discussion

We reviewed the evidence for PARG as a target for tissue protection. Because the outcome of any such evaluation depends strongly on the quality of the compounds used as tools, we undertook a thorough experimental reevaluation. Most evidence on the role of PARG in neuroprotection has been based on the use of gallotannin as a putatively selective inhibitor in cells. We present here evidence confirming that gallotannin is an inhibitor of PARG but also showing that it is not specific, and that it does not work in cells. We suggest that all data obtained with this compound so far should be carefully reevaluated.

Strong doubts on the specificity of the compound may arise already when one reads on the manufacturer's (Sigma) data sheet "...This product forms insoluble complexes with proteins. Insoluble precipitates are formed with albumin, gelatin, most alkaloids, and heavy metal salts [...] This product is used as a secondary fixative mixture with aldehydes for biological tissues, and also as a stain [...]". This unspecific binding to biomolecules and protein-staining by gallotannin may explain why Ying et al. show that gallotannin treatment leads to an apparent accumulation of PAR on Western blots (Ying et al., 2001). In our cellular fluorescent-based PAR assay, we observed the opposite, a clear decrease in PAR accumulation in HeLa cells treated with gallotannin. We also tested gallotannin for its ability to inhibit recombinant human PARG, and found that it was indeed an inhibitor of PARG as previously shown. This effect may be due to strong unspecific protein binding. This is corroborated by our finding that the IC_{50} of gallotannin in a PARG assay based on a cellular lysate is increased to about 150 μM. Unspecific protein binding by gallotannin possibly also inhibits NOs as described in cell-free systems (Chiesi and Schwaller, 1995; Kaneko et al., 1997; Ha et al., 2002). Moreover, the molecule might apparently "inhibit PARG", because it causes DNA strand breaks that activate PARP (Labieniec et al., 2003).

We established the cell death models used by Ying et al. to study the protective effect of gallotannin (Ying et al.). We could readily reproduce all astrocyte cell death models and confirm that PARP-1 inhibitors were indeed protective. Then, we tested gallotannin in these validated models. Gallotannin showed protection in the SIN-1 and H_2O_2 model but not in the MNNG model. We tried to find a protective effect of gallotannin in the MNNG model with extensive efforts, but all our data showed that gallotannin enhanced MNNG-induced cell death. Antioxidative effects of gallotannin best explain this set of data. This is consistent with our finding that gallotannin protected from H_2O_2 induced death but did not augment PAR accumulation. It was suggested previously that gallotannin works in the H_2O_2 model as a PARG inhibitor and not as an antioxidant, because *N*-acetyl cysteine (NAC) and *N*-tert butyl phenyl-nitrone (PBN) did not protect efficiently in the model. However, these compounds are structurally unrelated to tannins and are poor direct H_2O_2 scavengers. Tannins on the other hand have been shown to have strong H_2O_2-scavenging properties at low concentrations (Yokozawa et al., 1998). NAC and PBN have only one reactive group, whereas each galloyl-moiety on gallotannins has an aromatic ring with three hydroxyl groups in ortho-positions that can potentially react with hydroxyl radicals. The main gallotannin constituent, tetra-galloyl-glucose contains 13 phenolic hydroxyl groups, 12 of which are in ortho-positions. Instead of PBN or NAC, we tested two other polyphenols, catechin and quercetin containing five and six phenolic OH groups, with one or two groups in ortho-position, respectively. Both polyphenols protected substantially against H_2O_2-induced cell death, with quercetin (more hydroxyl groups) being almost as potent as gallotannin. None of these compounds has been implicated as PARG inhibitor. The strong antioxidant effects of gallotannin against H_2O_2 may also be due to its strong iron chelating effects (Lopes et al., 1999).

Finally, we demonstrated the radical scavenging effects of gallotannin directly against NO. We incubated gallotannin alone with two different NO donors, and we saw a concentration-dependent decrease in the accumulation of oxidised NO (nitrite) in the medium again showing the antioxidative properties of gallotannin. Further evidence for gallotannin as antioxidant and against cellular PARG inhibition comes from PAR accumulation assays in cells. We saw that PAR staining was decreased by gallotannin treatment of H_2O_2-activated cells. This is the opposite of what was previously reported and is inconsistent with PARG inhibition. If gallotannin acted as a PARG inhibitor, we would expect a delay in the decay of PAR, and thus a prolonged PAR accumulation, which was not observed. We

have previously shown that HeLa cell extracts contain a phosphodiesterase and/or ADP-ribose phyrophosphatase activity, which converts PAR and ADPR to AMP. This activity was not inhibited by gallotannin even at a concentration of 100 μM gallotannin (Keil et al., 2004). Therefore, the activity of other cellular PAR-hydrolysing enzymes could be responsible for the lack of a PAR accumulation in cells treated with specific PARG inhibitors.

Recently published data show that the IC_{50} value of the commercially available gallotannin preparation we used is around 150 μM in an assay testing PAR accumulation in cell extracts treated with nicked DNA (Keil et al., 2004). This suggests that high concentrations of gallotannin are required to inhibit PARG in complex biological system. The concentration used in the cell lysate-based PAR accumulation assay would cause massive cell death in our cellular system (data not shown). This, combined with the fact that we saw no effect in intact cells and no penetration in the Caco-2 monolayer could suggest that gallotannin is cell impermeable and mediates its effect extracellularly by lowering the concentrations of reactive oxygen species. In our cellular system, we observed protective effects from H_2O_2 at 5–10 μM, i.e., 10- to 15-fold below the IC_{50} values of gallotannin in cell extracts, again suggesting that gallotannin does not protect due to PARG inhibition.

GPI-16552 worked in the recombinant PARG assay with an IC_{50} value of about 50 μM, which is higher than what was previously reported (Lu et al., 2003). In addition, the maximal inhibitory effect was very modest, leaving about 60% of residual enzyme activity. In agreement with this, we found no evidence of protective properties in any of the viability assays in cells.

In conclusion, in our hands gallotannin is a PARG inhibitor in cell-free assays and acts as a strong antioxidant that can protect cells from oxidative stress. We did not find evidence for cellular PARG inhibition or protection from MNNG, although PARP-1 inhibitors were effective against this challenge. We found circumstantial as well as direct evidence (PAR and NO donor assay) that gallotannin works as an antioxidant. We also showed that GPI-16552 is a problematic tool compound. GPI-16552 worked with an IC_{50} value of 50 μM in a cell-free assay, but in the cell-based viability assays, no effect was seen. What does this imply for the role of PARG in neuroprotection? No PARG knockout mouse has yet been reported in the peer-reviewed literature, but $PARG^{-/-}$ ES cells have been reported to have increased sensitivity towards DNA damage (Masutani et al., 2003). A loss-of-function mutant in Drosophilae has shown to be lethal in the larval stage at 25 °C, but one-fourth developed to adult flies when they were kept at 29 °C. The flies showed progressive neurodegeneration with a strong accumulation of PAR, predominantly in the central nervous system (CNS). This suggests that PARG is the major PAR-degrading enzyme, at least in the CNS, and that the enzyme is indispensable (Hanai et al., 2004). The only clear conclusion on PARG that we can draw from our present work is that the currently used tool compounds are inadequate for a pharmacological evaluation of the role of PARG.

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