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RESEARCH PAPER

Mono-galloyl glucose derivatives are potent poly(ADP-ribose) glycohydrolase (PARG) inhibitors and partially reduce PARP-1-dependent cell death

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Background and purpose: Maintenance of poly(ADP-ribose) (PAR) polymers at homoeostatic levels by PAR glycohydrolase (PARG) is central in cell functioning and survival. Yet the pharmacological relevance of PARG inhibitors is still debated. Gallotannin, a complex mixture of hydrolysable tannins from oak gall, inhibits PARG but which of its constituents is responsible for the inhibition and whether the pharmacodynamic properties are due to its antioxidant properties, has not yet been established.

Experimental approach: A structure–activity relationship study was conducted on different natural and synthetic tannins/galloyl derivatives as potential PARG inhibitors, using a novel *in vitro* enzymic assay. Cytotoxicity was assayed in cultured HeLa cells.

Key results: Mono-galloyl glucose compounds were potent inhibitors of PARG, with activities similar to that of ADP-(hydroxymethyl) pyrrolidinediol, the most potent PARG inhibitor yet identified. When tested on HeLa cells exposed to the PAR polymerase (PARP)-1-activating compound 1-methyl-3-nitro-1-nitrosoguanidine (MNNG), 3-galloyl glucose weakly inhibited PAR degradation. Conversely, the more lipophilic, 3-galloyl-1,2-*O*-isopropylidene glucose, despite being inactive on the pure enzyme, efficiently prolonged the half-life of the polymers in intact HeLa cells. Also, PARG inhibitors, but not radical scavengers, reduced, in part, cell death caused by MNNG.

Conclusions and implications: Taken together, our findings identify mono-galloyl glucose derivatives as potent PARG inhibitors, and emphasize the active function of this enzyme in cell death.

British Journal of Pharmacology (2008) **155**, 1235–1249; doi:10.1038/bjp.2008.370; published online 22 September 2008

Introduction

Among the post-translational modification of proteins, poly(ADP-ribosyl)ation has recently emerged as a key event in the maintenance of cellular homoeostasis, as well as pathogenesis of various disorders. Poly(ADP-ribosyl)ation consists of the synthesis of polymers (up to 200 U) of poly(ADP-ribose) (PAR) from nicotinimade adenine dinucleotide (NAD$^+$), catalysed by a family of enzymes called PAR polymerases (PARPs) (Gagne *et al.*, 2006). PARP-1 is the oldest and best characterized member of the PARP family, and is responsible for almost 80% of cellular PAR formation, which typically takes place in the nucleus (Shieh *et al.*, 1998). Other non-nuclear PARPs have also been identified (Ame *et al.*, 2004), with tankyrase having a key function in mitotic spindle assembly (Chang *et al.*, 2005). Because of the high negative charge of the polymers, functions of numerous proteins are profoundly affected by PAR both by direct polymerization at specific glutamate residues and electrostatic interaction through non-covalent modification (D'Amours *et al.*, 1999; Pleschke *et al.*, 2000; Ahel *et al.*, 2008).

In the light of the impact of PAR on protein functions, intracellular contents of the polymers are kept under tight

control. This control is manifested by very rapid degradation (half-life of about 1 min) by PAR glycohydrolase (PARG), an enzyme exhibiting both exo- and endoglycosidic activity, and therefore releasing monomers of ADP-ribose or oligomers of PAR (Davidovich *et al.*, 2001; Gagne *et al.*, 2006). In contrast to the numerous PARPs involved in poly(ADP-ribosyl)ation, PARG is thought to be the main enzyme capable of degrading PAR. Indeed, although the polymer can be rapidly hydrolysed by phosphodiesterases *in vitro*, their function *in vivo* is still debated. In addition, an ADP-ribose hydrolase-like protein named ARH3 has been demonstrated to behave as a PARG (Oka *et al.*, 2006). PARG is ubiquitously expressed in mammalian cells, and nuclear localization/exportation signals in its amino-acid sequence allow fast nucleo-cytoplasmic shuttling and efficient PAR catabolism (Bonicalzi *et al.*, 2003). At present, various PARG isoforms have been identified, although only a single gene is present in human and rodent genome; PARG isoforms may arise from complex, alternative initiation of transcription, splicing and/or translation (Cortes *et al.*, 2004; Meyer *et al.*, 2007). Interestingly, the different isoforms have different kinetic properties, cellular localization and, possibly, functions (Cortes *et al.*, 2004; Gagne *et al.*, 2006; Meyer *et al.*, 2007). Regardless of the specific function(s) of PARG isoforms, complete suppression of the *parg* gene causes early embryonic lethality (Koh *et al.*, 2004). Conversely, mice selectively lacking the 110-kDa isoform of PARG (PARG$_{110}^{-/-}$) are viable, develop normally, despite being more susceptible to genotoxic and endotoxic shock, as well as to ischaemic brain injury (Cortes *et al.*, 2004; Cozzi *et al.*, 2006). PARG$_{110}^{-/-}$ mice, however, are protected from intestinal ischaemia and inflammatory bowel disease (Cuzzocrea *et al.*, 2005, 2007). Additional studies report a function for PARG in cell cycle regulation (Ohashi *et al.*, 2003), mitotic spindle assembly (Chang *et al.*, 2004), development (Hanai *et al.*, 2004; Koh *et al.*, 2004), differentiation (Di Meglio *et al.*, 2003) and cell death (Affar *et al.*, 2001; Ying *et al.*, 2001; Hanai *et al.*, 2004; Blenn *et al.*, 2006).

On this basis, a great deal of effort has been directed at developing potent chemicals able to selectively inhibit PARG activity. It is worth mentioning, indeed, that gene deletion studies are not always informative about the real significance of a protein to pharmacotherapy. Conversely, they may be misleading because gene deletion provides chronic rather than acute, inhibition of this protein's activity. At present, several studies have reported the inhibition of PARG by different compounds such as hydrolysable tannins, ethacridine, the ADP-ribose analogue ADP-(hydroxymethyl)-pyrrolidinediol (ADP-HPD), *N*-bis-(3-phenyl-propyl)9-oxo-fluorene-2,7-diamide (GPI 16552) (see Zhang and Li, 2002), and the modified PAR containing the etheno moiety (Shirato *et al.*, 2007). Although these compounds showed pharmaco-dynamic properties consistent with PARG inhibition (Ying *et al.*, 2001; Lu *et al.*, 2003; Rapizzi *et al.*, 2004; Cuzzocrea *et al.*, 2005), their use as tools able to inhibit PARG activity has been recently revisited. In particular, gallotannin (also known as tannic acid) exhibits nonspecific effects and appears unable to efficiently cross the plasma membrane, and GPI 16552 has proved to be of low potency (Falsig *et al.*, 2004).

Hydrolysable tannins are found in nature as multiple esters of gallic acid with glucose. The molecular weight of these compounds can vary from 332 (mono-galloyl glucose) to over 10 000 Da, with more than 500 different glucogalloyl derivatives being identified (Haslam, 1996). Although these compounds are of growing interest to consumers, food manufacturers and the pharmaceutical industry (Haslam, 1996; Maatta-Riihinen *et al.*, 2004; Romani *et al.*, 2005; Arapitsas *et al.*, 2007), the lack of pure, structurally defined and commercially available standards renders the study of their biological activities difficult. This is probably the main reason why various researchers use tannins available in the market (such as gallotannin) or home-made extracts (Khan *et al.*, 2000; Labieniec and Gabryelak, 2003; Ono *et al.*, 2004).

By means of an array of glucogalloyl derivatives obtained from the market or synthesized/isolated in our laboratories, in this study we aimed to gather structure–activity information on tannins and their pharmacophore for PARG inhibition, as well as furthering our understanding of the function of PARG in cell death.

Methods

NMR analysis

^{1}H NMR spectra at 200 and 400 MHz, and ^{13}C NMR spectra at 50 and 100 MHz, were obtained on a Varian Gemini 2000 spectrometer and a Varian Mercury Plus 400 spectrometer, respectively. For the NMR experiments, CDCl$_3$ and CD$_3$OD were used as solvents. Chemical shifts are reported in parts per million (p.p.m.) relative to the solvent non-deuterated residue. The following abbreviations are used: singlet (s), doublet (d), triplet (t), multiplet (m). High-resolution mass spectrometry (HRMS) electrospray ionization (ESI) was measured on LTQ Orbitrap-Ultimate 3000-(Finnigan LTQ), operated at the Interdepartmental Centre of Mass Spectrometry (CISM, Florence, Italy).

HPLC analysis

HPLC/DAD/MS analyses were carried out as reported earlier (Arapitsas *et al.*, 2007). Briefly, a HP 1100L liquid chromatograph equipped with a DAD detector (Agilent Technologies, CA, USA); linked to a HP 1100 MSD mass spectrometer with an API/ESI interface (Agilent Technologies, Santa Clara, CA, USA) was used. The mass spectrometer operating conditions were: gas temperature, 350 °C; nitrogen flow rate, 10.5 L min^{-1}; nebulizer pressure, 40 p.s.i.; quadrupole temperature, 40 °C and capillary voltage, 3500 V. A Luna C18(2) 250 mm × 4.60 mm, 5 µm (Phenomenex, Torrance, CA, USA) column was used, operating at 27 °C. HPLC/DAD/MS/MS analyses of tannic acid were carried out using a HP 1100L liquid chromatograph equipped with a DAD detector (Agilent Technologies) linked to a API 3000 linear triple quadruple mass spectrometer (Applied Biosystems, MDS Sciex, Toronto, ON, Canada) equipped with a pneumatically assisted ESI interface. Optimization for the parameters of the ESI/MS and ESI/MS/MS analyses of the gallotannin was performed by preparing a solution of tannic acid (10 µg mL^{-1}) in acetonitrile/water/formic acid (50:49.9:0.1

v/v/v). The solution was infused into the ES ionization source using a syringe pump at a flow rate 20 μL min^{-1}. The optimum conditions found for the analysis were: negative ion scan mode, nebulizer gas 6 p.s.i., curtain gas 8 p.s.i., collision gas 2.5×10^{-5} torr, ion spray voltage (IS) -4500 V, source temperature 200 °C, focusing potential -400 V, entrance potential -10 V, declustering potential -180 V, collision energy 60 V and collision cell exit potential -15 V. Nitrogen gas was used for curtain, collision and nebulizer. The enhanced MS scan mode in the mass range of 150–2100 U was performed. The operating system was Analyst 1.4. The column used was a 3.0 μm Discovery HS PEG, 10 cm × 2.1 mm i.d. (Supelco, Bellefonte, PA, USA), operating at 27 °C and with a flow rate of 0.2 mL min^{-1}. The mobile phase was a multistep linear solvent gradient system consisting of (A) 0.1% formic acid aqueous solution and (B) acetonitrile. The elution profile was: 3 min 95% A, then the solvent B was increased to 35% in 5 min, maintained for 2 min, further increased to 40% in 3 min and to 95% in 5 min. After 5 min, buffer B was decreased to 5% in 5 min.

Organic syntheses
Solvents were dried by usual laboratory techniques. Unless otherwise noted, all air and moisture sensitive reactions were performed under a positive nitrogen atmosphere.

3-(3,4,5-Tribenzyloxy)galloyl-1,2,5,6-di-O-isopropylidene-α-D-glucose. A mixture of 1,2,5,6-di-O-isopropylidene-α-D-glucose (250 mg, 1.0 mmol), 3,4,5-tribenzyloxy gallic acid (500 mg, 1.14 mmol) and 4-dimethylaminopyridine (250 mg, 1.20 mmol) in dry CH$_2$Cl$_2$ (20 mL) was refluxed for 1 h under nitrogen, then dicyclohexylcarbodiimide (160 mg, 1.20 mmol) was added and the mixture reacted with stirring at room temperature for 16 h. Later, the mixture was kept at -18 °C for 30 min and filtered through a pad of celite. The organic phase was washed with HCl 3% (1 × 20 mL), and brine (2 × 20 mL) dried over Na$_2$SO$_4$, concentrated and purified by flash chromatography (eluent petroleum ether (PE)/ethyl acetate (EtOAc) = 6:1) to obtain 3-(3,4,5-tribenzyloxy)galloyl-1,2,5,6-di-O-isopropylidene-α-D-glucose (510 mg, 80% yield) as a white powder. ^{1}H NMR (CDCl$_3$, 200 MHz) δ (p.p.m.): 1.29 (s, 4H, CCH$_3$), 1.34 (s, 4H, CCH$_3$), 1.44 (s, 4H, CCH$_3$), 1.58 (s, 4H, CCH$_3$), 4.04–4.24 (m, 2H, H$_{5+6}$), 4.30 (dd, $J = 7.6$ and 2.8 Hz, 1H, H$_4$), 4.59 (d, $J = 3.6$ Hz, 1H, H$_2$), 5.16 (s, 4H, 2 × CH$_2$Bn), 5.18 (s, 2H, CH$_2$Bn), 5.48 (d, $J = 2.8$ Hz, 1H, H$_3$), 5.94 (d, $J = 0.4$ Hz, 1H, H$_1$), 7.22–7.57 (m, 17H, CH$_{Ph}$). ^{13}C NMR (CDCl$_3$, 50 MHz) δ (p.p.m.): 22.2 (CH$_4$), 26.2 (CH$_4$), 26.7 (CH$_4$), 26.8 (CH$_4$), 67.0 (C$_6$), 71.1 (CH$_2$Bn), 72.4 (C$_3$), 75.0 (C$_5$), 76.4 (CH$_4$), 76.5 (CH$_4$), 79.7 (C$_2$), 83.1 (C$_4$), 104.9 (C$_1$), 109.1 (2 × C$_{Ph}$), 112.1 (2 × C$_{Ph}$), 124.1 (2 × CH of Bn), 127.1 (2 × CH of Bn), 127.8 (2 × CH of Bn), 128.0 (2 × CH of Bn), 128.2 (2 × CH of Bn), 128.3 (2 × CH of Bn), 136.2 (2 × C of Bn), 137.0 (2 × C of Bn), 142.5 (C$_{Ph}$), 152.2 (2 × C$_{Ph}$), 164.3 (CO).

3-Galloyl-1,2,5,6-di-O-isopropylidene-α-D-glucose. A solution of 3-(3,4,5-tribenzyloxy)galloyl-1,2,5,6-di-O-isopropylidene-α-D-glucose (900 mg, 0.76 mmol) in 10 mL of EtOAc/methanol

(MeOH) (3:1) was hydrogenated over Pd/C 10% (0.1 equiv) at room temperature with a hydrogen balloon. After 2 h, the mixture was filtered over a pad of celite and evaporated in vacuum: The grey–green glassy solid obtained was triturated with three portions of diethylether (Et$_2$O) and three portions of n-hexane and purified by flash chromatography (eluent EtOAc/PE/CH$_2$Cl$_2$ = 4:2:1) to obtain 3-galloyl-1,2, 5,6-di-O-isopropylidene-α-D-glucose (471 mg, 87% yield) as a colourless glassy solid. ^{1}H NMR (CD$_3$OD, 200 MHz) δ (p.p.m.): 1.27 (s, 4H, CCH$_3$), 1.32 (s, 4H, CCH$_3$), 1.39 (s, 4H, CCH$_3$), 1.51 (s, 4H, CCH$_3$), 3.58–3.84 (m, 2H, H$_6$), 3.84–3.99 (m, 1H, H$_5$), 4.28 (dd, $J = 8.2$ and 2.8 Hz, 1H, H$_4$), 4.62 (d, $J = 3.8$ Hz, 1H, H$_2$), 5.32 (d, $J = 2.8$ Hz, 1H, H$_3$), 5.95 (d, $J = 3.8$ Hz, 1H, H$_1$), 7.05 (s, 2H, H$_{Ph}$). ^{13}C NMR (CD$_3$OD, 50 MHz) δ (p.p.m.): 25.4 (CH$_4$), 26.0 (CH$_4$), 26.4 (CH$_4$), 27.0 (CH$_4$), 68.0 (C$_6$), 74.0 (C$_3$), 77.4 (C$_5$), 81.2 (C$_2$), 84.6 (C$_4$), 106.5 (C$_1$), 110.0 (2 × C$_{Ph}$), 113.2 (C$_{Ph}$), 139.9 (C$_{Ph}$), 146.4 (2 × C$_{Ph}$), 166.6 (CO). HRMS (ESI): calculated for C$_{19}$H$_{24}$O$_{10}$ (M + Na)$^+$, 435.1266; found, 435.1260.

3-Galloyl-1,2-O-isopropylidene-α-D-glucose (4). A solution of 3-galloyl-1,2,5,6-di-O-isopropylidene-α-D-glucose (100 mg, 0.24 mmol) was stirred for 2 h at room temperature in 10 mL of CHCl$_3$. The solution was concentrated and the crude residue was purified by trituration with three portions of Et$_2$O and three portions of n-hexane to obtain 3-galloyl-1,2-O-isopropylidene-α-D-glucose (4) (80 mg, 90% yield) as a glassy solid. ^{1}H NMR (CD$_3$OD, 400 MHz) δ (p.p.m.): 1.31 (s, 4H, CCH$_3$), 1.51 (s, 4H, CCH$_3$), 3.63 (dd, A part of an AB system, $J = 11.6$ and 5.6 Hz, 1H, H$_6$), 3.80 (dd, B part of an AB system, $J = 11.6$ and 2.8 Hz, 1H, H$_6$), 3.86–3.96 (m, 1H, H$_5$), 4.28 (dd, $J = 9.6$ and 2.8 Hz, 1H, H$_4$), 4.62 (d, $J = 3.6$ Hz, 1H, H$_2$), 5.37 (d, $J = 2.8$ Hz, 1H, H$_3$), 5.94 (d, $J = 3.6$ Hz, 1H, H$_1$), 7.07 (s, 2H, H$_{Ph}$). ^{13}C NMR (CD$_3$OD, 100 MHz) δ (p.p.m.): 26.4 (CH$_4$), 26.9 (CH$_4$), 65.2 (C$_6$), 70.0 (C$_3$), 77.5 (C$_5$), 80.1 (C$_2$), 84.5 (C$_4$), 106.6 (C$_1$), 110.2 (2 × C$_{Ph}$), 113.2 (C$_{Ph}$), 121.0 (C$_{Ph}$), 140.1 (C$_{Ph}$), 146.4 (C$_{Ph}$), 167.2 (CO). HRMS (ESI): calculated for C$_{16}$H$_{20}$O$_{10}$ (M + Na)$^+$, 395.0953; found, 395.0949.

α- and β-3-galloyl-D-glucose (5). A solution of 3-galloyl-1,2,5,6-di-O-isopropylidene-α-D-glucose (250 mg, 0.61 mmol) was stirred for 2 h at room temperature in CF$_3$COOH (2 mL). The solution was concentrated and the crude residue was purified by trituration with three portions of Et$_2$O and three portions of n-hexane to obtain 3-galloyl-D-glucose as a two-third mixture of α- and β-anomer (98 mg, 50% yield). ^{1}H NMR (CD$_3$OD, 400 MHz) δ (p.p.m.): 3.34–3.43 (m, 2H, H$_6$), 3.54–3.64 (m, 3H, H$_{β5+α6}$), 3.67–3.77 (m, 2H, H$_{β4+α5}$), 3.80 (dd, $J = 12.0$ and 2.4 Hz, 1H, H$_{β2}$), 3.87 (dd, $J = 12.0$ and 2.4 Hz, 1H, H$_{α2}$), 3.93 (m, 1H, H$_{α4}$), 4.61 (d, $J = 7.6$ Hz, 1H, H$_{α1}$), 5.10 (at, $J = 9.6$ Hz, 1H, H$_{α3}$), 5.17 (d, $J = 4.0$ Hz, 1H, H$_{β1}$), 5.39 (at, $J = 9.6$ Hz, 1H, H$_{β3}$), 7.13 (s, 2H, H$_{αPh}$), 7.13 (s, 2H, H$_{βPh}$). ^{13}C NMR (CD$_3$OD, 100 MHz) δ (p.p.m.): 62.4 (C$_s$), 62.6 (C$_s$), 66.9 (C$_s$), 70.0 (C$_s$), 70.1 (C$_s$), 72.4 (C$_s$), 73.0 (C$_s$), 74.8 (C$_s$), 77.3 (C$_s$), 78.0 (C$_s$), 79.4 (C$_s$), 94.1 (C$_{β1}$), 98.3 (C$_{α1}$), 110.4 (6 × C$_{Ph}$), 121.9 (C$_{Ph}$), 122.0 (C$_{Ph}$), 146.4 (2 × C$_{Ph}$), 168.3 (CO), 168.6 (CO). HRMS (ESI): calculated for C$_{13}$H$_{16}$O$_{10}$ (M + Na)$^+$, 356.0640; found, 356.0634.

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Extraction/isolation of hydrolysable tannins

The extraction of raspberries was achieved as reported earlier (Arapitsas et al., 2007). Briefly, 40 g of fresh raspberries in 300 mL of ethanol/water 70:30 was extracted for 18 h at room temperature. Later, the extract was filtered, concentrated, dissolved in ethanol/water 70:30 (20 mL) and directly analysed by HPLC/DAD/MS. The final volume of the extract was lyophilized and the powder, dissolved in 1 mL of ethanol/water 70:30, was loaded in a chromatographic column Sephadex LH-20 (30 cm × 1 cm). Stepwise elution was then performed using the following MeOH/acetone/H_2O mixture as mobile phase: 50 mL (90:0:10), 20 mL (70:20:10), 20 mL (30:50:20), 50 mL (0:80:20) and 50 mL (0:80:20). From this last fraction, 10 mg of sanguin H-6 was obtained. Hydrolysable tannins are absorbed to the stationary phase when methanol, ethanol and alcohols in general are used as mobile phase and they are released and eluted with aqueous acetone. For the isolation of 15 mg 1,2,3,4,6-pentagalloyl-β-D-glucose, and 5 mg trigalloyl-β-D-glucose, from 100 mg of tannic acid, the same column was used, but the mobile phase was isocratic acetone/water. The characterization of all compounds was made by HPLC/DAD/MS/MS and NMR analysis. To calculate the purity of each isolated and synthetic compound, 1 mg was separately dissolved in 1 mL of ethanol/water 70:30 and directly analysed by HPLC/DAD/MS.

PARG activity assay

^{3}H-PAR was synthesized in vitro according to Banasik et al. (1992). Briefly, 0.05 U of purified bovine PARP-1 was incubated at 37 °C in a final volume of 100 μL 50 mM Tris-HCl buffer (pH 7.4), containing 5 mM $MgCl_2$, 2 mM DL-dithiothreitol, 10 μg sonicated DNA, 0.5 mg mL^{-1} bovine albumin, 10 μM NAD and 1 μL ^{3}H-NAD (35.5 nmol). After 60 min, PARP-1 activity was blocked with 30 μM 6(5H)-phenanthridinone (PHE) and different units of PARG were added to the mixture in the presence or absence of potential or known PARG inhibitors. After 240 min incubation at 37 °C, the reaction was stopped by adding 1 mL of trichloroacetic acid (TCA) 10% (w/v). Then, the mixture was centrifuged (15 min at 12 000 g at 4 °C), and the pellet containing ^{3}H-PAR was washed twice with water and resuspended in 0.1 mM NaOH. Radioactivity was measured by scintillation counting (Tri-Carb 1900 TR; Packard, Meriden, CT, USA).

Cells and culture conditions

HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 10% foetal bovine serum and antibiotics. Cultures were brought to 50–70% confluence and exposed to 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) with or without pretreatment with different potential PARG inhibitors. Cell viability was evaluated by measuring lactate dehydrogenase release in the incubating media or reduction of methylthiazolyl tetrazolium as described earlier (Fossati et al., 2007).

Measurement of NAD, western blotting and immunocytochemistry

Measurement of intracellular NAD contents, western blotting, staining with Hoechst 33258 and immunocytochemistry

for PAR were performed as described earlier (Cipriani et al., 2005). HeLa cells were grown on coverslips and challenged with different compounds. Cells were then washed twice with phosphate-buffered saline (PBS) and fixed with cold PBS/4% paraformaldehyde for 10 min. Cells were incubated for 1 h in PBS/0.1% Triton X-100/bovine serum albumin (5 mg mL^{-1}) at room temperature, rinsed in PBS and incubated for 1 h with a polyclonal antiapoptosis-inducing factor (AIF) antibody (Santa Cruz Biotechnology, Tamecula, CA, USA) dissolved 1:200 in PBS/BSA (5 mg mL^{-1}). After washing with PBS, slides were incubated for 1 h with a Cy-3-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Westgrove, PA, USA) dissolved 1:200 in PBS/5 mg/mL BSA. Fluorescence was examined using a rhodamine filter (Ex 520 nm, Em >580 nm). Specificity of immune detection was established by the omission of the primary antibody.

Cell counting

In Hoechst 33258-stained cultures, 30 different microscopic fields (× 20) for each treatment were acquired by a charge coupled device camera. Cell nuclei were counted in each image and their number expressed as the mean ± s.e.mean.

Comet assay

DNA strand breaks were evaluated by the comet assay, as described earlier (Giovannelli et al., 2003). Briefly, centrifuged cells were suspended in agarose and layered on microscopic slides to be run through the comet assay. Microscopic analysis was carried out using a Labophot-2 microscope (Nikon, Tokyo, Japan) provided with epifluorescence. Each experimental point was run in duplicate and the images of 50 randomly chosen nuclei per slide were captured and analysed using a custom-made imaging software coupled with a charge coupled device camera (model C5985; Hamamatsu, Japan). The program calculated the total fluorescence distribution along the longer axis of the nucleus and the fluorescence distribution of the head and tail of the comet, respectively, for each image. Data were expressed as a percentage of total fluorescence migrated in the tail for each nucleus (% DNA in tail), a parameter linearly related to the number of DNA breaks. The mean % DNA in tail of 50 nuclei per slide was calculated, and the duplicate values were further averaged. The value of % DNA in tail obtained estimated the basal number of DNA strand breaks.

Statistical analysis

Evaluation of significant differences among groups was performed using the Student's t-test or ANOVA followed by Tukey's w-test. Statistical difference was considered for P-values <0.05.

Materials

MeOH, PE, EtOAc, dichloromethane (CH_2Cl_2), n-hexane and Et_2O were from Carlo Erba Reagenti SpA (Milano, Italy). Methyl gallate, CH_3CN, PHE, tritiated NAD (0.93 TBq mmol^{-1} 25.0 Ci mmol^{-1}) was from Perkin Elmer (Milan, Italy). Dicyclohexylcarbodiimide, N,N,-dimethyl-formamide HPLC grade, Hoechst 33258, bovine albumin,

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TCA, MNNG, MgCl$_2$, DL-dithiothreitol, 4-dimethylamino-pyridine, CF$_3$COOH, Pd/C 10%, Na$_2$SO$_4$, CDCl$_3$, CD$_3$OD, H$_3$PO$_3$, Pb(AcO)$_4$, tannic acid (**1**), gallic acid (**2**), methyl gallate (**3**), 1,2,5,6-di-*O*-isopropylidene-α-D-glucose and gallotannin were from Sigma-Aldrich (St Louis, MO, USA). Acetonitrile (HPLC grade) and formic acid were purchased from Aldrich Company Inc. (Milwaukee, WI, USA). 2,3-Digalloyl-α,β-D-glucose (**8**), 2,3-digalloyl-α,β-*O*-methyl-D-glucose (**9**), 2-galloyl-α,β-*O*-methyl-D-glucose (**7**), 3-galloyl-α, β-*O*-methyl-D-glucose (**6**), 2,3-hexahydroxydiphenoyl-α,β-D-glucose (**10**), 2,3-di(3-galloyl,4,5-dihydroxy-benzoyl)-α,β-D-glucose (**11**) and 3,4,5-tribenzyloxy gallic acid were prepared as described by Arapitsas *et al.* (2007). Calf thymus DNA (10 mg mL^{-1}) was from Invitrogen (San Diego, CA, USA). PARG bovine, PARP-1 and anti-PAR LP96-10 were from Alexis (Vinci Biochem, Vinci, Italy). ADP-HPD dehydrate ammonium salt was from Calbiochem (San Diego, CA, USA). Insta-gel plus was from Packard Instrument Co., Inc. (Downes Grove, IL, USA).

Results

Development of an in vitro *PARG activity assay*

Classic methods for screening of compounds as inhibitor of PARG activity are measurement of ^{32}P-PAR degradation by thin layer chromatography or quantitation of nuclear PAR levels by immunocytochemistry in cultured cells exposed to PARP-1-activating compounds (Tavassoli *et al.*, 1985; Tsai *et al.*, 1992; Falsig *et al.*, 2004; Keil *et al.*, 2004; Shirato *et al.*, 2007). Recently, a non-radiometric assay for high-through-put screening of PARG inhibitors has been reported (Putt and Hergenrother, 2004). In this study, we developed a very rapid and simple method to evaluate inhibition of PARG activity by measuring the hydrolytic activity of purified bovine PARG on ^{3}H-PAR. As shown in Figure 1a, incubation of pure PARP-1 with ^{3}H-NAD in the presence of sonicated DNA (containing histones) and bovine albumin led to the incorporation of radioactivity in TCA-precipitated proteins. Radioactivity incorporation was time dependent, tended to reach a plateau at 60-min incubation and was completely prevented by the presence of the PARP-1 inhibitor 6(5*H*)-phenanthridinone (Banasik *et al.*, 1992) (Figure 1a). It is noted that the addition of PHE after 60 min blocked the residual incorporation of radioactivity, with the latter remaining stable for at least 3 h after PARP-1 inhibition (Figure 1a). These results indicate that radioactivity was incorporated into proteins by PARP-1, and that these polymers were stable under our experimental settings. Conversely, addition of pure PARG to the mixture after 60 min reduced the radioactivity measured in the TCA-precipitated pellet. When PARG was added together with PHE, an almost complete reduction of precipitable radioactivity was recorded (Figure 1b), suggesting extensive degradation of PAR, by the exo/endoglycosidic activity of PARG. PAR degradation by PARG was concentration dependent, reaching a maximiun at 0.05 U (Figure 1c).

Inhibition of PARG activity by gallolyl-glucose derivatives

Gallotannin is classically used as a PARG inhibitor despite being a mixture of different compounds. Indeed, as shown in
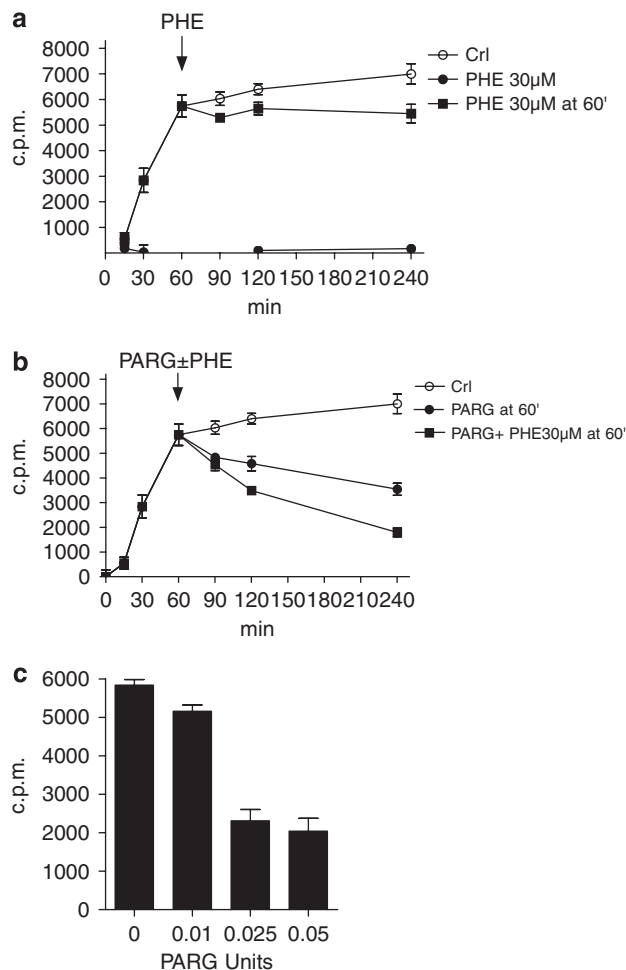

Figure 1 Synthesis and degradation of poly(ADP-ribose) (PAR) by pure poly(ADP-ribose) polymerase (PARP-1) and poly(ADP-ribose) glycohydrolase (PARG). (**a**) Time-dependent incorporation of radio-activity from ^{3}H-NAD into trichloroacetic acid (TCA)-precipitated proteins in the absence or presence of 30 μM phenanthridinone (PHE; a PARP-1 inhibitor) at time 0 or 60 min. (**b**) Effect of pure PARG (0.05 U) added after 60 min on incorporation of radioactivity from ^{3}H-NAD into TCA-precipitated proteins in the absence or presence of 30 μM PHE. (**c**) PARG-dependent reduction of radioactivity incorporation is concentration dependent. Each point/column represents the mean ± s.e.mean of three (**a**, **b**) and two (**c**) experiments conducted in duplicate.

Figure 2a, chromatographic analysis coupled to mass spectrometry of solubilized gallotannin revealed the presence of gallic acid and various galloyl glucose derivatives containing up to 12 galloyl residues (see legend of Figure 2a). Although the presence of methyl-gallate, di- and tetra-galloyl glucose in the gallotannin mixture has been reported (Romani *et al.*, 2006; Arapitsas *et al.*, 2007), under our experimental conditions their concentrations were below detection limits. We therefore next evaluated the effect of the galloyl-glucose derivatives on PAR degradation by PARG in the above-mentioned enzymatic assay. We tested galloyl-glucose derivatives with increasing complexity (Figures 2b and 3a) to understand which constituent of gallotannin is mostly responsible for PARG inhibition, as well as to gather information on their structure–activity relationship. To this
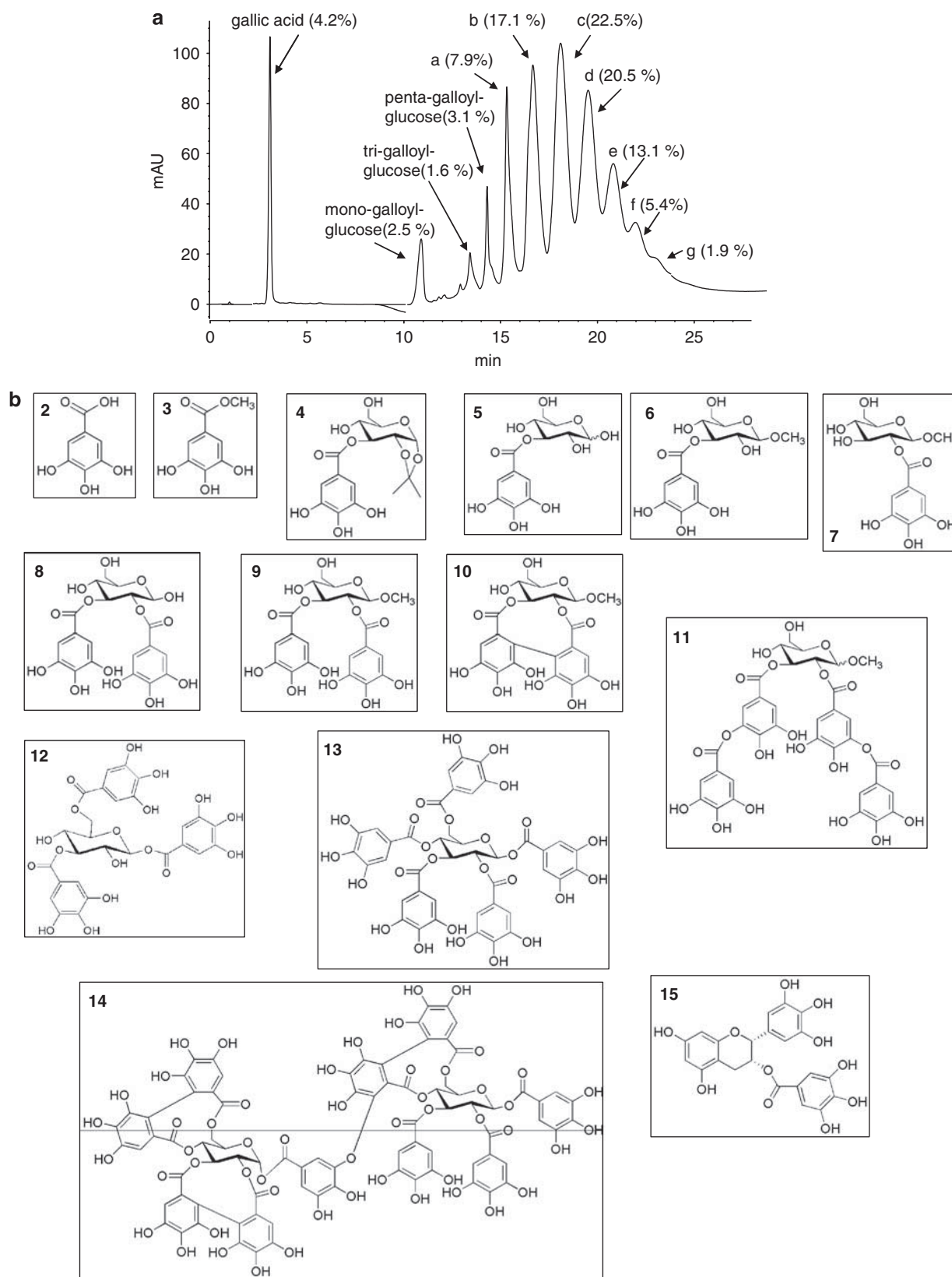
PARG inhibition by galloyl-glucose
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Figure 2 Chemical constituents of gallotannin and other galloyl-containing molecules tested as poly(ADP-ribose) glycohydrolase (PARG) inhibitors. (**a**) Representative HPLC/DAD chromatographic profile of gallotannin, reported at 280 nm. The presence and % of the different galloyl derivatives are shown. a–g represent the esa-, epta-, octa-, ena-, deca-, endeca-, dodeca- and galloyl glucose derivatives, respectively. (**b**) Structures of galloyl derivatives tested as PARG inhibitors.

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end, we tested compounds available in the market as well as molecules synthesized *ex novo* or from precursors extracted from gallotannin (Figures 2 and 3a, and see Methods). As shown in Figure 3b, gallotannin (**1**, see also Figure 3a) led to a 25% inhibition of PARG activity when tested at 10 μM, in good agreement with its reported IC_{50} of about 30 μM (Tsai *et al.*, 1992). Gallic acid (**2**), a component of gallotannin, had no effects on the enzyme activity or its derivative methyl-gallate (**3**) (Figures 2 and 3b), synthesized to increase the lipophilicity of gallic acid. Similarly, 3-galloyl-1,2-O-iso-propylidene glucose (**4**), intermediate of the synthesis of 3-galloyl-glucose, did not inhibit PARG at 10 and 100 μM

(Figure 3b and not shown). An inhibition of about 65% of PAR degradation was obtained with 3-galloyl-α,β-D-glucose (**5**), 3-galloyl-O-methyl-α,β-D-glucose (**6**) and 2-galloyl-O-methyl-α,β-D-glucose (**7**) when tested at 10 μM (Figure 3b). These compounds showed IC_{50} values on PARG activity of 0.95 ± 0.02, 7.1 ± 0.05 and 7.2 ± 0.03 μM, respectively. It is noted that their inhibitory potency was in the same range of that of ADP-HPD (**16**), that under our experimental settings had an IC_{50} of 3.2 ± 0.08 μM (Figure 3c).

By increasing the number of gallic residues from one to two such as in compounds (**8**) and (**9**) (Figure 2), the inhibitory activity on PARG decreased (Figure 3b). Neither

a **Compounds tested as PARG inhibitors**

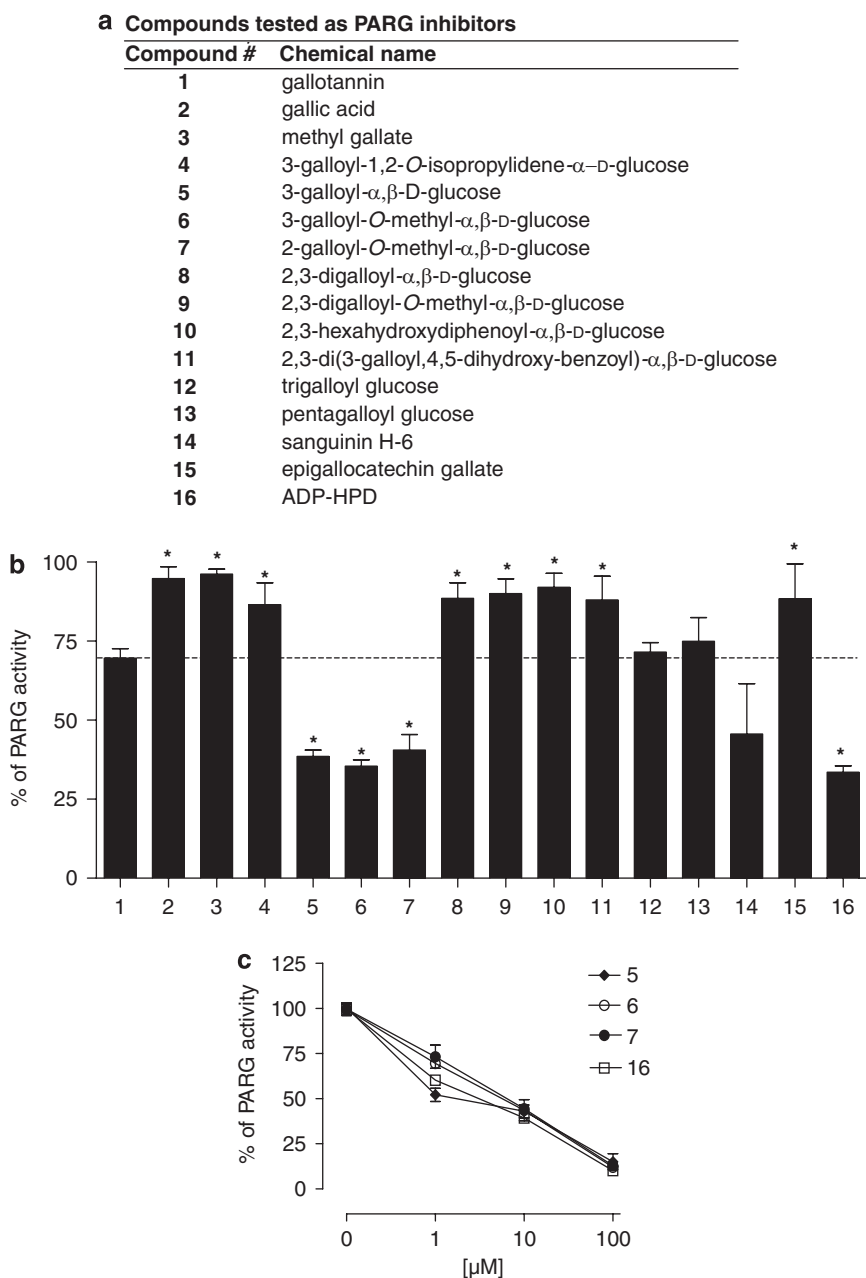| Compound #	Chemical name
1	gallotannin
2	gallic acid
3	methyl gallate
4	3-galloyl-1,2-O-isopropylidene-α–D-glucose
5	3-galloyl-α,β-D-glucose
6	3-galloyl-O-methyl-α,β-D-glucose
7	2-galloyl-O-methyl-α,β-D-glucose
8	2,3-digalloyl-α,β-D-glucose
9	2,3-digalloyl-O-methyl-α,β-D-glucose
10	2,3-hexahydroxydiphenoyl-α,β-D-glucose
11	2,3-di(3-galloyl,4,5-dihydroxy-benzoyl)-α,β-D-glucose
12	trigalloyl glucose
13	pentagalloyl glucose
14	sanguinin H-6
15	epigallocatechin gallate
16	ADP-HPD

Figure 3 Mono-galloyl glucose derivatives are potent poly(ADP-ribose) glycohydrolase (PARG) inhibitors. (**a**) List with chemical names and numbers of the compounds tested as PARG inhibitors. (**b**) Histogram showing the relative inhibitory potency of the compounds tested at 10 μM on PARG activity. (**c**) Inhibitory activity on PARG of compounds (**5**), (**6**), (**7**) and (**16**). In (**b**, **c**), each column/point represents the mean ± s.e.mean of three experiments conducted in duplicate. *$P < 0.05$ vs no. 1 (ANOVA + Tukey's *post hoc* test).

the presence of a C–C bond between the two gallic acid residues (hexahydroxydiphenoyl group) as in compound (**10**) nor the presence of depsidic moieties as in compound (**11**) led to significant PARG inhibition (Figure 3b). Conversely, an increase in gallic residues bound to glucose as in tri- and pentagalloyl glucose (**12** and **13**, respectively) augmented the inhibitory potency on PARG when compared with digalloyl substitutes (Figure 3b). We also tested the complex hydrolysable tannin sanguinin H-6 (**14**), which, at 10 μM, caused about 50% inhibition (Figure 3b). The gallic acid-containing, antioxidant compound epigallocatechin gallate (**15**) had no effects on PARG activity when tested at 10 μM, whereas at the same concentrations, the potent PARG inhibitor ADP-HPD (**16**) reduced the PAR-hydrolysing activity of PARG by ∼70% (Figure 3b).

Effects of mono-galloyl glucose derivatives on PAR content in intact cells

Among the galloyl glucose derivatives, the most potent were 3-galloyl-glucose, 3-galloyl-*O*-methyl-glucose and 2-galloyl-glucose (**5**, **6** and **7**, respectively). We next evaluated whether these chemicals could inhibit PARG activity in intact cells. To this end, we used HeLa cells, a cell line characterized by massive synthesis of PAR on exposure to alkylating agents, and rapid polymer degradation by PARG (Rossi *et al.*, 2002; Cipriani *et al.*, 2005). Cells were exposed to the methylating agent MNNG and PAR contents were evaluated by western blotting or immunocytochemistry. As shown in Figure 4a, PAR content significantly increased 5 min after exposure to MNNG, and rapidly returned to basal level after 15 and 30 min. Incubation with 3-galloyl-glucose (**5**) 30 min before and during the challenge with MNNG did not provoke any apparent change in the reduction of PAR contents at 10 and 15 min (Figure 4a). In keeping with previous reports (Alvarez-Gonzalez and Althaus, 1989), these findings indicate that PAR degradation by PARG is efficiently activated on polymer synthesis. They also suggest that 3-galloyl-glucose (**5**), despite being a potent inhibitor *in vitro* (Figures 2b and c), exerts weak inhibition of PAR degradation in cultured cells. Given the high hydrophilicity of 3-galloyl-glucose, we reasoned that, akin to gallotannin (Falsig *et al.*, 2004), its low inhibitory potency in intact cells could be ascribed to a low permeability through the plasma membrane. To address this issue, we took advantage of the availability of 3-galloyl-1,2-*O*-isopropylidene glucose (**4**), a protected precursor in the synthesis of 3-galloyl-glucose containing a isopropyldene group (Figure 2). We reasoned that such a compound should have higher lipophilicity and hence a facilitated passage through the plasma membrane. Also, once inside the cell, it could be readily transformed into 3-galloyl-glucose (**5**) by nonspecific hydrolases. In good agreement with this hypothesis, western blotting showed that 3-galloyl-1,2-*O*-isopropylidene glucose (**4**), despite being inactive *per se* (Figure 3b), increased the basal contents of PAR when added to the culture medium at concentrations of 10 or 100 μM. Notably, 3-galloyl-1,2-*O*-isopropylidene glucose (**4**) also counteracted the reduction of PAR content at 15 and 30 min after MNNG exposure (Figure 4b).

These findings suggest that 3-galloyl-1,2-*O*-isopropylidene glucose (**4**) is a cell-permeable precursor of the potent PARG inhibitor 3-galloyl-glucose. Immunocytochemistry confirmed these data by showing that the nuclear content of PAR was higher in cells exposed 15 and 30 min to MNNG plus 3-galloyl-1,2-*O*-isopropylidene glucose (**4**), relative to those challenged with MNNG only (Figure 4c). In keeping with the basal levels of PAR in resting HeLa cells (indicative of ongoing poly(ADP-ribosyl)ation, first lane of Figure 4b), exposure to PHE significantly increased intracellular NAD content (Figure 4d). The latter also increased on exposure to 3-galloyl-1,2-*O*-isopropylidene glucose (**4**) (Figure 4d), indicating that the increased content of PAR found in cells challenged with the compound (first three lanes of Figure 4b) is not due to PARP-1 activation. Conversely, we ascribe the increased contents of NAD in cells exposed to 3-galloyl-1,2-*O*-isopropylidene glucose (**4**) to the ability of the latter to inhibit PARG and the ensuing inhibition of PARP-1 activity because of excessive auto-(ADP-ribosyl)ation (Davidovich *et al.*, 2001; Rapizzi *et al.*, 2004). In contrast to the results with PHE, however, 3-galloyl-1,2-*O*-isopropylidene glucose (**4**) had no effect on intracellular NAD depletion prompted by MNNG (Figure 4e), suggesting that it does not affect hyper-poly(ADP-ribosyl)ation on DNA damage.

Effect of gallotannin, 3-galloyl-1,2-O-isopropylidene glucose or radical scavengers on cell death induced by MNNG

It has been reported that PARG inhibition/suppression exerts protection against cell death in models of PARP-1 hyperactivation (Ying *et al.*, 2001; Lu *et al.*, 2003; Cuzzocrea *et al.*, 2005, 2007; Patel *et al.*, 2005; Blenn *et al.*, 2006). However, the study by Falsig *et al.* (2004) demonstrated that the cytoprotective effects of the PARG inhibitor gallotannin (**1**) were related to its antioxidant properties and not to the inhibition of PARG. To gather further information on PARG inhibition and cell death, HeLa cells were exposed to gallotannin (**1**) or 3-galloyl-1,2-*O*-isopropylidene glucose (**4**) in the presence or absence of MNNG and cell death was evaluated 24 h later by means of methylthiazolyl tetrazolium reduction or lactate dehydrogenase release assays. As reported earlier (Cipriani *et al.*, 2005), MNNG-dependent cell death was completely prevented by two inhibitors of PARP-1 such as PJ34 and PHE (not shown), indicating that the enzyme's hyperactivation is causative in cell death. The results in Figure 5 show that, under our conditions, both PARG inhibitors caused cytotoxicity when present in the incubating media for 1, 3 and 6 h. Yet cell death evaluated 23 h after 1 h exposure to MNNG was in part reduced by gallotannin (**1**) or 3-galloyl-1,2-*O*-isopropylidene glucose (**4**) when present in the incubating media for 3 or 6 h (Figures 5b and c) but not 12 or 24 h (not shown). However, a 12 h exposure to the PARG inhibitors afforded protection from MNNG when cell death was evaluated at this time point (Figure 6a). To understand whether partial prevention of cell death showed by the PARG inhibitors were due to radical scavenging, we tested the effects of the well-known radical scavengers 4-hydroxy-TEMPO and epigallocatechin gallate (**15**) on PARP-1-dependent cell death triggered by MNNG.
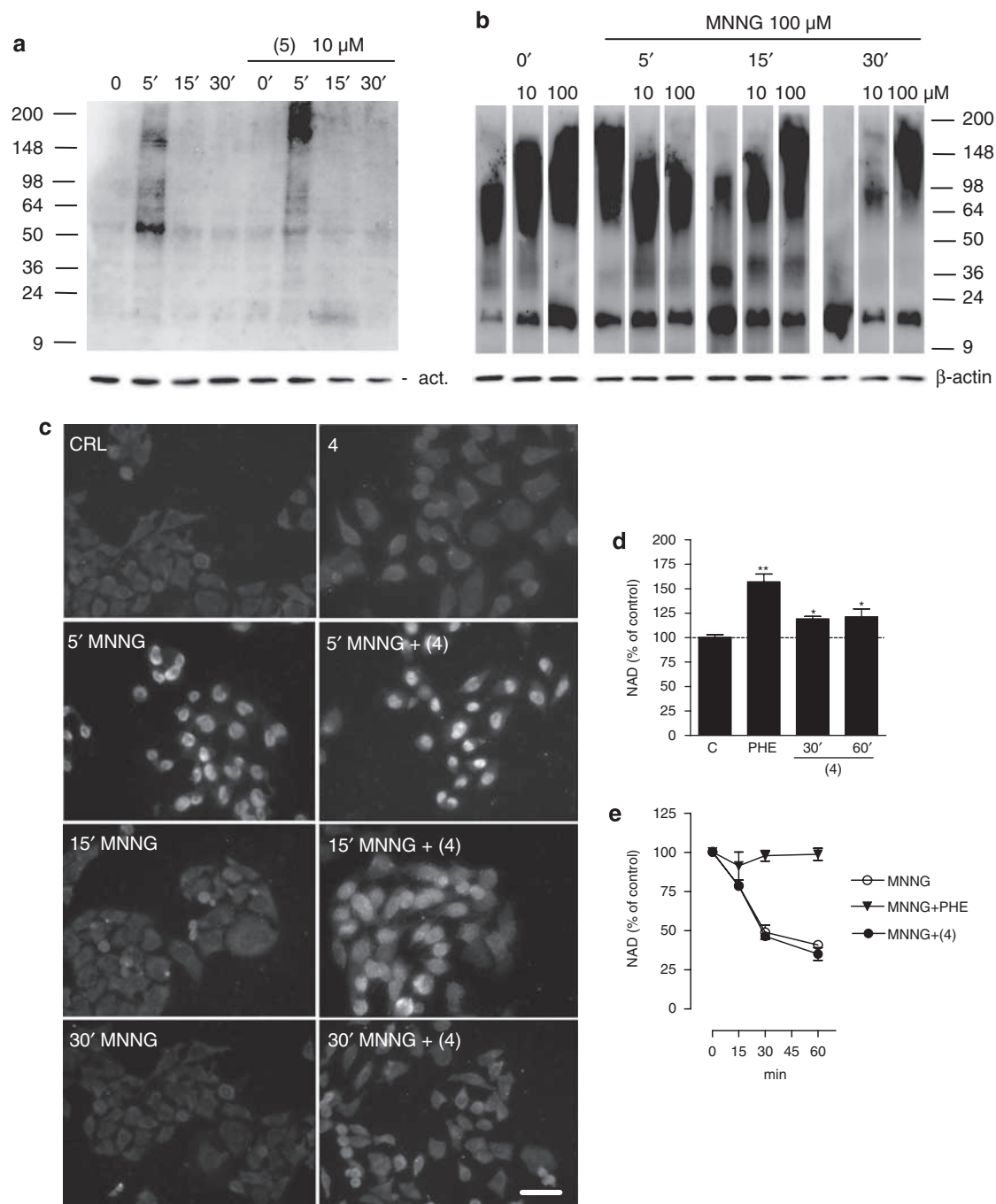
Figure 4 Effects of mono-galloyl glucose on poly(ADP-ribose) glycohydrolase (PARG) activity in intact cells. (**a**) HeLa cells were exposed to the poly(ADP-ribose) polymerase (PARP-1)-activating compound 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) (100 μM) in the absence or presence of 10 μM 3-galloyl-glucose (**5**) and poly(ADP-ribose) (PAR) levels were evaluated by western blotting at different times. β-Actin is shown as a loading control. (**b**) HeLa cells were exposed to the PARP-1-activating compound MNNG (100 μM) in the absence or presence of 3-galloyl-1,2-O-isopropylidene glucose (**4**) (10 or 100 μM) and PAR levels were evaluated by western blotting at different time points. β-Actin is shown as a loading control. Compound (**4**) was pre-incubated for 30 min. (**c**) Immunocytochemical evaluation of intracellular PAR contents in cells exposed to MNNG in the absence or presence of 3-galloyl-1,2-O-isopropylidene glucose (**4**, 100 μM). (**d**) Nicotinimade adenine dinucleotide (NAD) levels in cells exposed 30 min to 30 μM 6(5H)-phenanthridinone (PHE) or 30 and 60 min to 100 μM 3-galloyl-1,2-O-isopropylidene glucose (**4**). (**e**) NAD contents in cells exposed to 100 μM MNNG in the presence or absence of PHE (30 μM) or 3-galloyl-1,2-O-isopropylidene glucose (**4**, 100 μM). A blot/experiment representative of two (**a–c**) or three (**d**) is shown. Bar = 20 μm. Columns are the mean ± s.e.mean of two experiments (**e**). *$P < 0.05$, **$P < 0.01$ vs control (C) (ANOVA + Tukey's *post hoc* test).

Notably, the presence of the two antioxidants in the culture medium had no effects on MNNG-dependent cell death evaluated 11 h after 1 h exposure to the alkylating agent (Figure 6a). These findings taken together suggest that PARG inhibition rather than radical scavenging underlies cytoprotection by gallotannin (**1**) or 3-galloyl-1,2-O-isopropylidene glucose (**4**) during PARP-1 hyperactivation. Analysis of cell morphology revealed that cells exposed for 1 h to MNNG
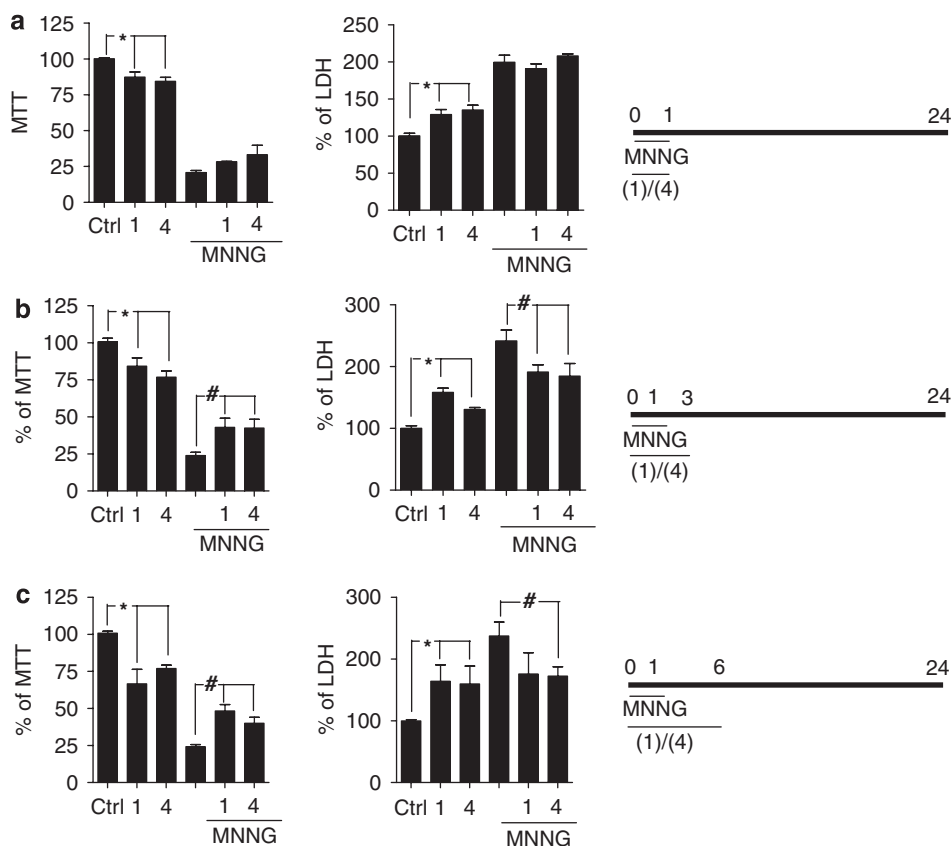
Figure 5 Effect of gallotannin or 3-galloyl-1,2-*O*-isopropylidene glucose on cell death triggered by 24 h exposure to 1-methyl-3-nitro-1-nitrosoguanidine (MNNG). Cells were exposed to 100 μM gallotannin (**1**) or 100 μM 3-galloyl-1,2-*O*-isopropylidene glucose (**4**) in the presence or absence of 100 μM MNNG for 1 h and cell death was evaluated 24 h later by means of methylthiazolyl tetrazolium (MTT) reduction or lactate dehydrogenase (LDH) release assays. Both PARG inhibitors were present in the incubating media for 1 (**a**), 3 (**b**), 6 h (**c**) (see time lines on right of **a–c**). Bars are the mean ± s.e.mean of three experiments conducted in duplicate. *$P < 0.05$ vs control (ctrl), #$P < 0.05$ vs MNNG (ANOVA + Tukey's *post hoc* test).

acquired a round appearance and detached from the plate 11 h later. In contrast, cells exposed to gallotannin (**1**) or 3-galloyl-1,2-*O*-isopropylidene glucose (**4**) during MNNG and for the following 11 h were still adherent to the plate. However, they appeared unhealthy being swollen and with cytoplasmic vacuoles. Cells challenged with gallotannin (**1**) or 3-galloyl-1,2-*O*-isopropylidene glucose (**4**) for 12 h in the absence of MNNG did not show morphological abnormalities (Figure 6b). Cell counting confirmed these findings by showing that the number of cells was reduced almost to 50% after exposure to MNNG (100 μM; 12 h) but was unchanged in cultures exposed to the two PARG inhibitors alone or in combination with MNNG (Figure 6c). By means of the comet assay, we also checked whether 3-galloyl-1,2-*O*-isopropylidene glucose (**4**) induced DNA damage. As shown in Figures 6d and e, we found that MNNG (100 μM) but not the galloyl glucose derivative triggered significant DNA breaks. To further investigate cytoprotection from PARP-1 over-activation afforded by gallotannin (**1**) or 3-galloyl-1,2-*O*-isopropylidene glucose (**4**), we next analysed their effects on two typical events occurring during PARP-1-dependent cell death, namely, release of AIF from mitochondria and chromatin condensation. In keeping with prior work (Cipriani *et al.*, 2005), AIF immunoreactivity lost the typical punctate distribution present in resting cells and appeared diffuse in the cytosol and nucleus (indicating mitochondrial release of AIF) 3 h after exposing HeLa cells to a 1 h challenge to MNNG. AIF immunoreactivity was not significantly modified in cells exposed for 4 h to gallotannin (**1**) or 3-galloyl-1,2-*O*-isopropylidene glucose (**4**). However, mitochondrial AIF release was prevented in part in cells exposed to MNNG (1 h) plus gallotannin (**1**) or 3-galloyl-1,2-*O*-isopropylidene glucose (**4**) (Figure 7a). Cell nuclei appeared shrunken often showing pyknosis and chromatin condensation 8 h after exposure to MNNG (1 h). The presence of gallotannin (**1**) or 3-galloyl-1,2-*O*-isopropylidene glucose (**4**) did not affect nuclear morphology. Yet nuclear alterations of MNNG-challenged cells were partially prevented by gallotannin (**1**) or 3-galloyl-1,2-*O*-isopropylidene glucose (**4**) present in the medium for the 8-h period. Specifically, the two compounds prevented nuclear condensation triggered by MNNG but nuclei were still shrunken with irregular surfaces (Figure 7b).

Discussion and conclusions

In this study, we investigated the effects of different hydrolysable tannins with increasing complexity on PARG activity. By means of a novel assay for PARG activity, we
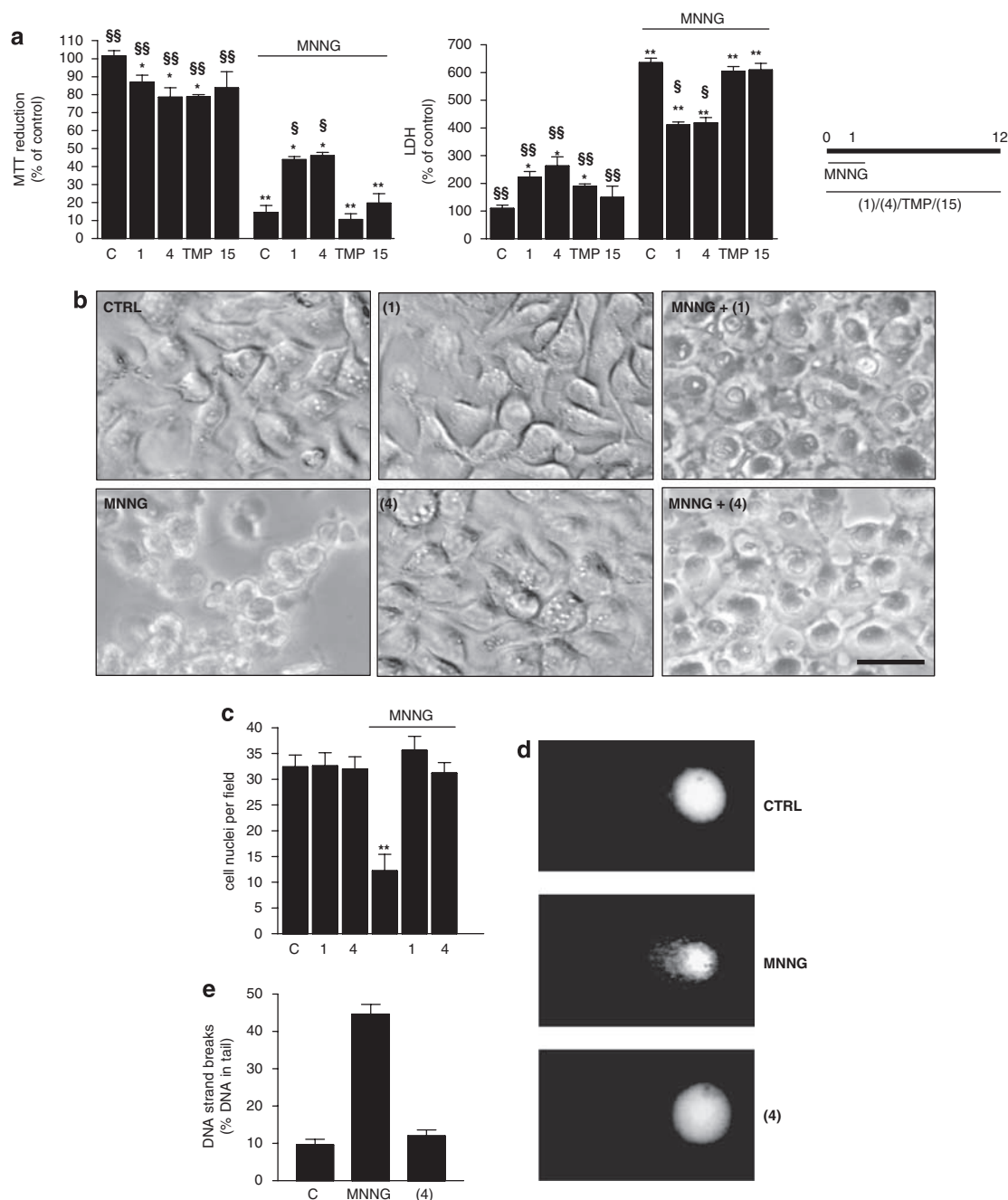
Figure 6 Effect of gallotannin, 3-galloyl-1,2-O-isopropylidene glucose, 4-hydroxy-TEMPO (TMP) or epigallocatechin gallate on cell death triggered by 12h exposure to 1-methyl-3-nitro-1-nitrosoguanidine (MNNG). (**a**) HeLa cells were exposed to 100 μM gallotannin (**1**), 100 μM 3-galloyl-1,2-O-isopropylidene glucose (**4**), 1 mM TMP or 100 μM epigallocatechin gallate (**15**) for 12h and cell death was evaluated by methylthiazolyl tetrazolium (MTT) reduction and lactate dehydrogenase (LDH) release. Columns represent the mean ± s.e.mean of three experiments conducted in duplicate. *$P < 0.05$, **$P < 0.01$ vs ctrl. $^{§}P < 0.05$, $^{§§}P < 0.01$ vs MNNG (ANOVA + Tukey's *post hoc* test). (**b**) Phase-contrast micrograph visualization of cells exposed with or without 100 μM MNNG for 1 h in the presence or absence of 100 μM gallotannin (**1**) or 100 μM 3-galloyl-1,2-O-isopropylidene glucose (**4**). (**c**) Cell counting of cultures exposed to 100 μM gallotannin (**1**) or 100 μM 3-galloyl-1,2-O-isopropylidene glucose (**4**) in the presence or absence of MNNG. **$P < 0.01$ vs ctrl (ANOVA + Tukey's *post hoc* test). (**d**) Cell nuclei visualized with the comet assay of cells exposed for 30 min to MNNG or 3-galloyl-1,2-O-isopropylidene glucose (**4**) both at 100 μM. (**e**) Measurement of DNA damage (% of DNA damage in tail) in cells treated as in (**d**). Bars represent the mean ± s.e.mean of three (**a, c**) and two (**e**) experiments. Images are representative of three (**b**) and two (**d**) experiments. Bar = 20 μm.

report that mono galloyl glucose derivatives inhibit the enzyme with IC$_{50}$ values in the same range of that of ADP-HPD. It must be pointed out that ADP-HPD is reported to have an IC$_{50}$ of 0.12 μM (Slama *et al.*, 1995b) or 0.33 μM (Koh *et al.*, 2003a), approximately 10- to 25-fold lower than that reported in this study. At present, we do not know the reason for this apparent discrepancy. It is worth noting, however, that the efficiency of the hydrolytic activity of PARG
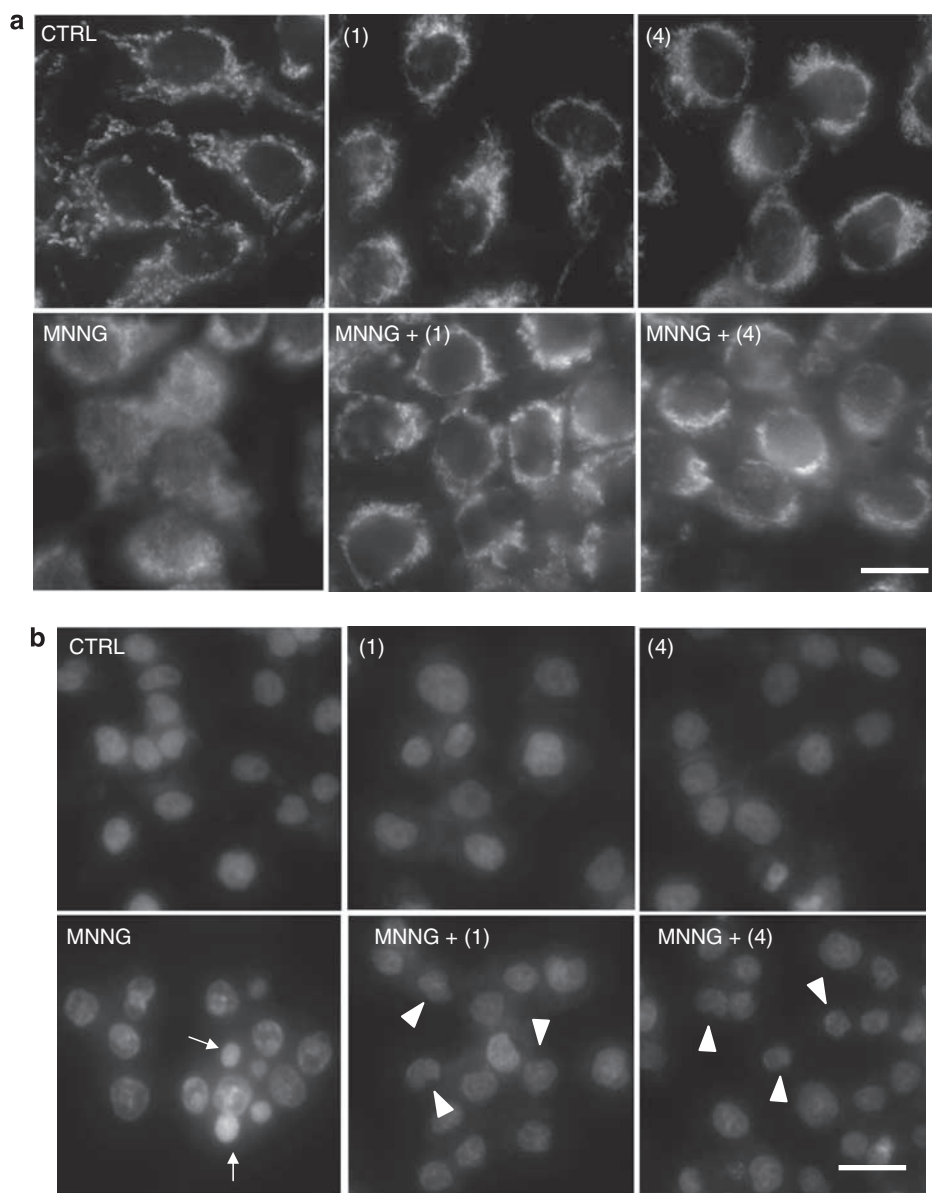
Figure 7 Effect of gallotannin (**1**) or 3-galloyl-1,2-*O*-isopropylidene glucose (**4**) on mitochondrial apoptosis-inducing factor (AIF) release and nuclear morphology of cells exposed to 1-methyl-3-nitro-1-nitrosoguanidine (MNNG). (**a**) AIF immunostaining of cells under control conditions or 4 h after exposure to 100 μM MNNG for 1 h in the absence or presence of 100 μM gallotannin (**1**) or 100 μM 3-galloyl-1,2-*O*-isopropylidene glucose (**4**). The effect of the two compounds alone is shown. Note the loss of punctate staining and AIF redistribution into the cytoplasm and nucleus in MNNG-exposed cells. (**b**) Hoechst 33258 staining of nuclei of cells under control conditions or 8 h after exposure to 100 μM MNNG for 1 h in the absence or presence of gallotannin (**1**) or 3-galloyl-1,2-*O*-isopropylidene glucose (**4**) both at 100 μM. The effect of gallotannin (**1**) or 3-galloyl-1,2-*O*-isopropylidene glucose (**4**) alone is also shown. Note nuclear shrinkage, pyknosis and chromatin condensation (arrows) in MNNG-exposed cells, and partial prevention of these nuclear alterations by 3-galloyl-1,2-*O*-isopropylidene glucose (**4**). Arrowheads point to partially shrunken nuclei with irregular surfaces. Images are representative of three experiments. Bar = 20 μm (**a**) and 10 μm (**b**).

depends on the molecular weight and structure of the polymer. In particular, PARG targets the terminal ADP-ribose residue as well as the endoglycosidic bond and branching points of PAR. Also, degradation of the polymers starts with a rapid hydrolysis until approximately 50% of the chains are consumed, and then turns into a slow distributive degradation leading to the release of monomers of ADP-ribose. It is noted that the degradation of large polymers (>20 ADP-ribose units) is higher than that of short ones (Hatakeyama

et al., 1986), and ADP-HPD more efficiently inhibits PARG when processing high molecular weight polymers (Ramsinghani *et al.*, 1998). Given that we do not know the length of the polymers synthesized in our *in vitro* assay, we consider that the higher value of the IC_{50} for ADP-HPD reported here could be ascribed to structural differences of the substrate when compared with that used in previous reports (Slama *et al.*, 1995b; Koh *et al.*, 2003a). These interpretations could also help reconcile the discrepancies

reported for the potency of the PARG inhibitor GPI 16552 (Lu *et al.*, 2003; Falsig *et al.*, 2004). Nevertheless, evidence that ADP-HPD is considered the most potent PARG inhibitor currently developed (Zhang and Li, 2002; Gagne *et al.*, 2006) underscores the relevance of our findings, and points to 3- and 2-galloyl glucose as tools useful for pharmacologically modulating PARG activity. The presence of mono-galloyl glucose in gallotannin (Figure 2a) also suggests that these compounds represent the most potent inhibitory components of this natural extract.

Our findings suggest that mono-galloyl glucose derivatives, although characterized by low plasma membrane permeability (see Falsig *et al.*, 2004), can inhibit PAR degradation in intact cells when designed as more lipophilic pro-drugs. Structure–activity relationship analysis of tannins as PARG inhibitors has not been conducted yet. Our finding that mono-galloyl glucose inhibits PARG suggests that this simple structure is, or represents for the most part, the pharmacophore of the complex hydrolysable tannins such as nobotannin (Ying *et al.*, 2001) or sanguinin H-6 (this study). Also, evidence that the methylation of the anomeric carbon of glucose does not affect the inhibitory potency of the compounds suggests that an easy glucose ring opening is not essential for PARG inhibition. These findings, taken together, further our understanding of the mechanisms of inhibition of PARG by tannins, and allow development of more potent PARG inhibitors. In addition, we report the interesting finding that mono-, tri- and pentagalloyl-glucose moieties inhibit PARG, whereas the digalloyl-glucose derivatives are very weak inhibitors. Even though we do not have an explanation for this result, it is worth noting that the binding site(s) of tannins on PARG is (are) unknown. In this regard, studies from Jacobson's group suggest that interaction of PARG with PAR occurs through multiple binding sites that can be differently occupied by chemical inhibitors (Slama *et al.*, 1995a, b; Ramsinghani *et al.*, 1998; Koh *et al.*, 2003a, b). Selective occupancy of one of these putative sites has been postulated to underlie the unusual, non-competitive inhibition of PARG by the product analogue ADP-HPD (Slama *et al.*, 1995b). On this basis, tannins might interact with the extended PAR-binding domain of PARG in a complex manner, and their binding ability to the multiple ADP-ribosyl-binding sites might not be directly proportional to their gallic acid residues. In other words, digalloyl glucose could occupy the enzyme pocket in a manner still permitting binding and hydrolysis of PAR, whereas mono-, tri-, penta- and complex tannin could interact with a number of binding sites which, because of their spatial distribution, more efficiently prevent binding of the polymer to PARG. An additional hypothesis is that tannins interact with PAR rather than PARG. Indeed, both polymers and tannins are highly reactive molecules and their mutual interaction might lead to non-hydrolysable ADP-ribose chains.

Pharmacological inhibition of PARG reportedly affords cytoprotection (Ying *et al.*, 2001; Lu *et al.*, 2003; Cuzzocrea *et al.*, 2005, 2007; Patel *et al.*, 2005; Blenn *et al.*, 2006). Accordingly, we show that both gallotannin (**1**) and 3-galloyl-1,2-*O*-isopropylidene glucose (**4**) reduce MNNG-triggered cell death evaluated 24 h later when present in the incubating media for the first 3 or 6 h, but not 12 or 24 h.

Yet a 12 h exposure to the PARG inhibitors partially protects from MNNG toxicity, if cell viability is evaluated at this time point. These findings, together with evidence that both compounds are endowed with intrinsic cytotoxicity, suggest that cytoprotection by PARG inhibition was reduced and, after certain exposure times, was actually abolished, because of the direct toxicity of the compounds. Theoretically, partial protection and intrinsic cytotoxicity might be due to intracellular accumulation of PAR (Figure 4b), a toxic polymer (Andrabi *et al.*, 2006) potentially hindering the cytoprotective effects of PARG inhibition. Overall, this study indicates that acute PARG inhibition is beneficial, reducing or delaying the apoptotic events triggered by PARP-1 (Figure 7), but becomes detrimental when prolonged. This interpretation is in line with prior study showing cell death originating from genetic suppression of PARG (Cortes *et al.*, 2004; Hanai *et al.*, 2004; Koh *et al.*, 2004; Andrabi *et al.*, 2006; Cozzi *et al.*, 2006; Yu *et al.*, 2006). Finally, evidence that radical scavengers do not alter MNNG-dependent cell death (Figure 6a) is at odds with the hypothesis that cytoprotection by tannins is due to their antioxidant properties (Falsig *et al.*, 2004). Conversely, our data strengthen the claims that, under certain experimental settings, the pharmacodynamic effects of tannins are due to PARG inhibition (Tsai *et al.*, 1992; Ying *et al.*, 2001; Dumitriu *et al.*, 2004; Keil *et al.*, 2004; Rapizzi *et al.*, 2004; Cuzzocrea *et al.*, 2005, 2007; Patel *et al.*, 2005; Blenn *et al.*, 2006).

In conclusion, this study identifies two mono-galloyl glucose derivatives as the simplest tannins able to inhibit PARG. Knowledge of the kinetic mechanisms of the enzyme, as well as deeper structural insights into its binding domain(s), is needed to better comprehend its mode of action and develop potent and selective inhibitors. The latter will help clarify the enzyme's function under different pathophysiological conditions as well as the therapeutic relevance of its pharmacological inhibition.

Acknowledgements

Conflict of interest

The authors state no conflict of interest.

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