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Novel isoquinolinone-derived inhibitors of poly(ADP-ribose) polymerase-1: pharmacological characterization and neuroprotective effects in an *in vitro* model of cerebral ischemia

Alberto Chiarugi, Elena Meli, Maura Calvani, Roberta Picca, Roberto Baronti, Emidio Camaioni, Gabriele Costantino, Maura Marinozzi, Domenico E. Pellegrini-Giampietro, Roberto Pellicciari & Flavio Moroni

Dipartimento di Farmacologia Preclinica e Clinica, Viale Pieraccini 6, Università di Firenze, 50139 Firenze (A.C., E.M., M.C., R.P., R.B., D.E.P.G., F.M.) and Dipartimento di Chimica e Tecnologia del Farmaco, Università di Perugia,Via del Liceo 1, 06127 Perugia (E.C., G.C., M.M., R.P.), Italy.

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Corresponding author: Prof. Flavio Moroni

Dipartimento di Farmacologia Preclinica e Clinica

Università di Firenze

Viale Pieraccini, 6

50139 Firenze, Italy

Telephone: +39-055-4271 266

Telefax: +39-055-4271 280

E-mail: flavio.moroni@unifi.it

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Abbreviations: DPQ, 3,4-dihydro-5-[4-(1-piperidinyl)buthoxyl)]-1(*2H*)-isoquinolinone; LDH, lactate dehydrogenase; OGD, oxygen-glucose deprivation; 5OH-DIQ, 3,4-dihydro-5-hydroxy-1(*2H*)-isoquinolinone; PARP, poly(ADP-ribose) polymerase; PND, 6(*5H*)-phenanthridinone; TIQ-A, thieno[2,3-c] isoquinolin-5-one.

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Abstract

Excessive activation of poly(ADP-ribose) polymerase-1 (PARP-1), a nuclear enzyme catalyzing the transfer of ADP-ribose units from NAD to acceptor proteins, induces cellular energy failure by NAD and ATP depletion and has been proposed to play a causative role in a number of pathological conditions including ischemia/reperfusion injury. In this study, we used an *in vitro* enzyme activity assay to characterize a series of newly synthesized isoquinolinone derivatives as potential PARP-1 inhibitors. Several compounds displayed powerful inhibitory activity: thieno[2,3-c] isoquinolin-5-one (TIQ-A) displayed a submicromolar IC_{50} of 0.45 ± 0.1 μM, whereas the 5-hydroxy and 5-methoxy TIQ-A derivatives had IC_{50}s of 0.39 ± 0.19 and 0.21 ± 0.12 μM, respectively. We then examined the neuroprotective effects of the newly characterized compounds in cultured mouse cortical cells exposed to 60 min oxygen and glucose deprivation (OGD). When PARP-1 inhibitors were present in the incubation medium during OGD and the subsequent 24 h recovery period, they significantly attenuated neuronal injury. TIQ-A provided neuroprotection even when added to the culture 30 min after OGD and was able to reduce the early activation of PARP induced by OGD, as detected by flow-cytometry. When the IC_{50} values observed in the PARP-1 activity assay for selected compounds were compared with their IC_{50}s for the neuroprotective activity, a significant correlation ($r = 0.93$, $P < 0.01$) was observed. Our results suggest that TIQ-A and its derivatives are a new class of neuroprotectants that may be helpful in studies aimed at understanding the involvement of PARP-1 in physiology and pathology.

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Poly(ADP-ribosyl)ation is a covalent post-translational modification of proteins catalyzed by a family of enzymes involved in numerous cellular processes including DNA repair and telomere replication (Smith, 2001). Poly(ADP-ribose) polymerase-1 (PARP-1, E.C. 2.4.2.30), a nuclear protein activated by DNA strand breaks, is probably the most studied member of this family. Its activation represents an immediate cellular response to DNA damage and facilitates maintenance of genome integrity (D'Amours et al., 1999; Shall and de Murcia, 2000; Herceg and Wang, 2001). PARP-1 catalyzes the transfer of multiple ADP-ribose units (up to 200) from NAD^+ to a variety of substrates including PARP-1 itself, histones and other target proteins involved in nuclear functioning, DNA repair and gene expression (de Murcia et al., 1994; Chiarugi, 2002). It has been suggested that excessive activation of this enzyme may cause cellular NAD^+ and ATP deficiency, eventually leading to energy failure and necrotic cell death (Cosi et al., 1994; Zhang et al., 1994; Ha and Snyder, 1999; Herceg and Wang, 2001). In addition, a role of PARP-1 in apoptosis-inducing factor-mediated cell death has been reported (Yu et al., 2002), demonstrating a key role of this enzyme in the activation of the apoptotic program. Accordingly, PARP-1 inhibitors have been shown to be of therapeutic value in a number of pathological conditions such as diabetes (Pieper et al., 1999; Soriano et al., 2001), inflammation (Szabó and Dawson, 1998), haemorrhagic shock (McDonald et al., 2000; Liaudet et al., 2000), and hepatic (Bowes and Thiemermann, 1998) or cerebral (Endres et al., 1997; Ha and Snyder, 2000; Moroni et al., 2001) ischemia.

To study the biological role of PARP-1, a range of strategies such as antisense oligonucleotides, gene deletion, expression of dominant negative forms (D'Amours et al., 1999) and RNA interference (Gan et al., 2002) have been used. In addition, several classes of competitive PARP-1 inhibitors have been reported. Relevant examples include benzamide (Banasik et al., 1992), 3,4-dihydro-5-hydroxy-1(*2H*)-isoquinolinone (5OH-DIQ) (Arundel-

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Suto et al., 1991), 3,4-dihydro-5-[4-1(1-piperidinyl)buthoxy]-1(*2H*)-isoquinolinone (DPQ) (Suto et al., 1991) and 6(*5H*)-phenantridinone (PND) (Banasik et al., 1992) (Fig. 1). Recently, it has been proposed that adenosine, inosine or hypoxanthine may act as endogenous PARP-1 inhibitors (Virag and Szabo, 2001). The disclosure of a number of high resolution crystallographic structures of the PARP-1 catalytic site complexed with various inhibitors has represented a major breakthrough in the understanding of the molecular basis of PARP-1 inhibitor binding (Ruf et al, 1996; Ruf et al. 1998). Indeed, all the crystallized inhibitors show a conserved pattern of interactions which include: *i)* a hydrogen bonding interaction between the mandatory amido group of inhibitors in an *anti* disposition and Gly863, *ii)* a π-π interaction between an aromatic ring of inhibitors and Tyr907-Try896. In order to extend the available information on the structure-activity relationship for competitive PARP-1 inhibitors, we have recently reported a thorough QSAR/docking study based on the analysis of as many as 46 competitive PARP-1 inhibitors (Costantino et al., 2001). As a continuation of this work, and with the aim of characterizing new PARP-1 inhibitors, we now report the effects of a new series of isoquinolinone derivatives on PARP-1 enzymatic activity and in an *in vitro* model of cerebral ischemia [oxygen and glucose deprivation (OGD) in primary cultures of mixed cortical cells].

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Materials and Methods

Materials

Glutamate and PND were purchased from Sigma-Aldrich (Milan, Italy). [Adenine-2,8-^{3}H]NAD (1-5 Ci/mmol) was from Du Pont/NEN (Milan, Italy). Lactate dehydrogenase (LDH) activity was quantified using the Cytotoxicity Detection Kit (LDH) from Roche Diagnostics (Monza, MI, Italy). DPQ and partially purified (90%) bovine PARP-1 were purchased from Alexis (Läufelfingen, Switzerland). The polyclonal antibody directed against poly(ADP-ribose) (PAR) (LP96-10) was from Alexis Corp. Italia (Vinci, Italy). The anti-glial fibrillary acidic protein (GFAP) fluoresceine isothiocyanate (FITC)-conjugated antibody and the phycoerythrin-conjugated goat anti-rabbit IgG were purchased from Molecular Probes Europe (Leiden, The Netherlands).

5OH-DIQ and the compounds shown in Fig. 2, i.e. 3,4-dihydro-5-mercapto-isoquinolin-1(*2H*)-one (compound 1), [3,4-dihydro-5-oxo-isoquinolin-1(2H)-one]-benzoic ester (compound 2), 3,4-dihydro-5-ethynyl-isoquinolin-1(2H)-one (compound 3), 3,4-dihydro-5-hydroxy-isoquinolin-2(*1H*)-one (compound 4), [3,4-dihydro-5-oxo-isoquinolin-2(*1H*)-one]-benzoic ester (compound 5); 6-hydroxy-2,3,4,5-tetrahydro-benzo[c]azepin-1-one (compound 6); 5-(4-piperidin-1-yl-but-2-ynyloxy)-3,4-dihydro-2*H*-isoquinolin-1-one (compound 7), 5-(5-piperidin-1-yl-pent-1-ynyl)-3,4-dihydro-2*H*-isoquinolin-1-one (compound 8); 6-(4-piperidin-1-yl-butoxy)-2,3,4,5-tetrahydro-benzo[*c*]azepin-1-one (compound 9), 2-methyl-8-(4-piperidin-1-yl-butoxy)-3*H*-quinazolin-4-one (compound 10), thieno[2,3-c] isoquinolin-5-one (TIQ-A), 9-hydroxythieno[2,3-c] isoquinolin-5(*4H*)-one (compound 11), and 9-methoxythieno[2,3-c]isoquinolin-5(*4H*)-one (compound 12) were synthesized as described elsewhere (Pellicciari et al., 2003). The structures of the new compounds were determined by ^{1}H-NMR and ^{13}C-NMR with a Bruker AC 200 MHz

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spectrometer. All derivatives displayed an HPLC purity >95%, as detected with a Shimadzu LC-10 workstation equipped with an SPD-10A UV-Vis detector and using a Lichrospher Si60 (250 x 5 mm, 5 μm) column for compounds 5OH-DIQ and 1 through 10 or a Lichrospher 100 RP-18 (250 x 5 mm, 5 μm) column for compounds TIQ-A, 11 and 12. The flow rate was 0.8 mL/min, the detection was carried out at 210 and 254 nm, and the mobile phase was a mixture of CH_2Cl_2/MeOH (Lichrospher Si60) or H_20/MeCN + 0.1% TFA (Lichrospher 100 RP-18).

Assay of PARP-1 activity and inhibition studies

PARP-1 activity was evaluated as previously described (Moroni et al., 2001). Briefly, the enzymatic reaction was carried out in a final volume of 100 μl consisting of: 20 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 5 mM dithiothreitol, 20 μg sonicated calf thymus DNA, 0.2 μCi [adenine-2,8-^{3}H]NAD, and 0.13 U of partially purified bovine PARP-1. To evaluate the inhibitory potency of the tested compounds, different concentrations were dissolved in 50% dimethyl sulfoxide and then 10 μl were added to the enzymatic reaction. An equal amount of the vehicle was also added to the control samples. The mixture was incubated at 37°C for 1 h and the reaction terminated by adding 500 μl of 50% trichloroacetic acid followed by brief centrifugation. After two gentle washes of the pellet with 1 ml distilled water, the radioactivity incorporated from [adenine-2,8-^{3}H]NAD into proteins was evaluated by liquid scintillation spectrometry.

Oxygen-glucose deprivation in cortical cell cultures

Cultures of mixed cortical cells containing both neuronal and glial elements were prepared, used at 14 days *in vitro* and exposed to oxygen-glucose deprivation (OGD) as previously described in detail (Pellegrini-Giampietro et al., 1999a; Pellegrini-Giampietro et al., 1999b). Briefly, culture medium was replaced by a glucose-free balanced salt solution

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saturated with 95% N_2/5% CO_2 and heated to 37°C. Multiwells were then sealed into an airtight incubation chamber equipped with inlet and outlet valves and 95% N_2/5% CO_2 was blown through the chamber for 10 min to ensure maximal removal of oxygen. The chamber was then sealed and placed into the incubator at 37°C for 60 min. OGD was terminated by removing the cultures from the chamber, replacing the exposure solution with oxygenated medium and returning the multiwells to the incubator under normoxic conditions. The extent of neuronal death was assessed 24 h later. In this system, 60 min OGD induced a neuronal damage that was approximately 75% of the maximal degree of neuronal injury achieved by exposing the cultures for 24 h to 1 mM glutamate. OGD-induced cell injury was quantitatively evaluated by measuring the amount of LDH released from injured cells into culture media 24 h following exposure to OGD, as previously described (Pellegrini-Giampietro et al., 1999a; Pellegrini-Giampietro et al., 1999b). The LDH level corresponding to complete neuronal death (with no glial death) was determined for each experiment by assaying sister cultures exposed to 1 mM glutamate for 24 h. Background LDH release was determined in control cultures not exposed to OGD and subtracted from all experimental values. The resulting value correlated linearly with the degree of cell loss estimated by observation of cultures under phase-contrast microscopy or under bright-field optics following 5 min incubation with 0.4% trypan blue, which stains debris and nonviable cells.

Measurement of PARP activity by flow-cytometry

PARP activity was evaluated in cortical cells exposed to OGD by cytofluorimetric measurement of PAR formation according to Affar et al. (1999), with minor modifications. Briefly, 1 h after OGD neuronal cultures were detached using 0.05% trypsin for 5 min at 37°C in PBS, washed with cold PBS and fixed with 4% paraformaldehyde at room temperature. Fixed cells were permeabilized with 0.2 % NP-40, washed with PBS, saturated with PBS-MT

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(PBS containing 5% non-fat powdered milk and 0.1% Tween 20) for 1 h and then incubated overnight at 4°C with anti-PAR (1:100) and FITC-conjugated anti-GFAP (1:100) antibodies diluted in PBS-MT. After several washes with PBS-MT, cells were incubated with a phycoerythrin-conjugated anti-rabbit IgG (1:50) for 30 min. The cell suspension (containing both neurons and astrocytes) was analyzed using a flow cytometer (Coulter XL, Coulter Cytometry, Hialeah, FL, USA). By means of appropriate electronic gates, neuron-related events (PAR positive neurons) were sorted by subtracting FITC-labeled cells (GFAP-positive astrocytes) from phycoerythrin-positive cells.

Statistical analysis

Concentration-response curves of PARP inhibitors were analyzed and IC_{50} values were calculated with the Prism software package (GraphPAD Software, San Diego, CA). Statistical significance of differences between results was evaluated by performing ANOVA followed by Tukey's *w* test for multiple comparisons. Spearman's *r* correlation coefficient was computed between IC_{50} values of experimental compounds on the inhibition of PARP activity and the IC_{50} values on OGD-induced LDH release. The P value was calculated using a two-tailed test.

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Results

Effects of isoquinolinone derivatives on PARP-1 activity

In a previous report, we showed that the widely used PARP-1 inhibitor benzamide (Fig. 1) was able to reduce the enzyme activity with an IC_{50} of 30 ± 4 μM when tested in an *in vitro* assay system (Moroni et al., 2001). 5OH-DIQ can be considered as a constrained benzamide analog, where the 5-position corresponds to the 3-position of benzamide and the amido moiety is restrained in an *anti* disposition (Fig. 1). In agreement with previous observations (Arundel-Suto et al., 1991), 5OH-DIQ was a moderately potent PARP-1 *in vitro* with an IC_{50} of 17 ± 4 μM (Table 1, Fig. 3A). A thiol (compound 1 in Fig. 2) or a benzoyloxy (compound 2) group inserted in 5-position reduced the inhibitory potency of 5OH-DIQ, whereas an acetylene group (compound 3) did not modify its activity (Table 1, Fig. 3A). Compounds 4 and 5, which are endowed with an 'inverse' *syn* disposition of the amido group, had no significant PARP-1 inhibitory activity up to a concentration of 100 μM. Compound 6, characterized by having the amido group constrained into a seven-membered ring, displayed a reduced potency with respect to the corresponding 3,4-dihydroisoquinolinone derivative (Table 1, Figs. 3A), thus suggesting that the incorporation of the amido moiety into a seven-membered ring may result in a non-optimal interaction with the active site residues. It should be noted, however, that a different series of potent PARP-1 inhibitors incorporating a seven-membered lactame moiety into a tricyclic structure has recently been reported (Canan Koch et al., 2002). The different conformation induced on the seven-membered ring by the tricyclic with respect to the bicyclic scaffold may explain the difference.

Effects of DPQ derivatives on PARP-1 activity

DPQ (Fig. 1) is another potent PARP-1 inhibitor (Banasik et al., 1992; Eliasson et al., 1997; Moroni et al., 2001), with an IC_{50} of 2.2 ± 0.5 μM (Table 1, Fig. 3B). A series of

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analogues were synthesized by modifying either the buthoxy-piperidine side chain or the dihydroisoquinolinone ring (Fig. 2). Compounds having the buthoxy-piperidine side chain conformationally constrained with a triple bond inserted in the 2'- position (compound 7) or in the place of the phenolic oxygen (compound 8) were synthesized, but their PARP-1 inhibitory activity was lower than that of DPQ (Table 1, Fig. 3B). As described for compound 6, when the six member ring structure of dihydroisoquinolinone of DPQ was changed into an seven-membered ring (compound 9), the inhibitory potency decreased. Conversely, when it was changed into a quinazolin-4(*3H*)-one structure (compound 10), PARP-1 inhibitory activity was not affected (Table 1 and Fig. 3B).

Effects of PND derivatives on PARP-1 activity

PND (Fig. 1) is a PARP-1 inhibitor significantly more potent than benzamide (Banasik et al., 1992; Moroni et al., 2001). We evaluated the effect of the isosteric replacement of ring C of PND with a 5-membered thiophene ring. TIQ-A (Fig. 2) turned out to be a very potent PARP-1 inhibitor, with an IC_{50} of 0.45 ± 0.1 μM (Table 1, Fig. 3C). Further manipulation of TIQ-A led to the 5-hydroxy and 5-methoxy derivatives (compounds 11 and 12), two molecules exhibiting a similar potent inhibitory activity (IC_{50} values: 0.39 ± 0.19 and 0.21 ± 0.10 μM, respectively).

Neuroprotective effects of isoquinolinone derivatives against OGD neurotoxicity in murine cortical cell cultures

PARP-1 inhibitors provide neuroprotection in models of post-ischemic neurodegeneration (Eliasson et al., 1997; Ha and Snyder, 2000; Moroni et al., 2001). To investigate whether the potency of the newly characterized PARP-1 inhibitors correlated with their neuroprotective effects, we used cultured murine cortical cells exposed to OGD as an *in*

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vitro model of cerebral ischemia. Phase-contrast microscopy revealed that exposure to a high concentration (1 mM) of glutamate for 24 h induced complete neuronal cell death but no injury in the underlying glial layer, as well as a substantial (approximately four-fold the basal levels) release of LDH into the bathing medium (data not shown, see for details: (Pellegrini-Giampietro et al., 1999a). Exposure to OGD for 60 min produced an intermediate level of neuronal damage: the release of LDH was approximately 75% of that observed by exposing the cultures to 1 mM glutamate. None of the tested PARP-1 inhibitors produced any significant increase in the release of LDH when added alone into the incubation media (not shown).

A number of PARP-1 inhibitors displaying an IC_{50} < 100 μM in the *in vitro* enzymatic assay were added to the incubation medium during OGD exposure and the subsequent 24 h recovery period. As previously shown (Moroni et al., 2001), benzamide, PND and DPQ reduced OGD injury in a concentration-dependent manner, with IC_{50}s of 103 ± 12, 10.6 ± 2 and 4 ± 0.6 μM, respectively. In a similar manner, 5OH-DIQ attenuated neuronal death following OGD with an IC_{50} of 71 ± 14 μM, whereas compound 8 displayed an IC_{50} of 63 ± 12 μM. The thieno-derivatives TIQ-A and compound 11 markedly reduced OGD-induced neuronal death with IC_{50}s of 0.15 ± 0.01 and 0.2 ± 0.1 μM, respectively. Figure 4A reports the neuroprotective effects of these PARP-1 inhibitors at a concentration of 100 μM. Although compounds 3 and 12 inhibited PARP at concentrations lower than 100 μM, they were not tested against OGD because they could not be completely dissolved in the lower concentration of dimethylsulfoxide (0.1 % *vs* 5 %) required for cell culture studies as compared to the *in vitro* enzymatic assay. When the IC_{50} values observed in the PARP-1 activity assay for these compounds were compared with their IC_{50}s for the reduction of OGD-induced LDH release, a significant correlation was found ($r = 0.89$, $P < 0.0014$, Fig. 4B). In keeping with the relative inhibitory potencies on PARP-1, TIQ-A and compound 11 were

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significantly more potent than their parent compound PND in reducing OGD-induced LDH release (Fig. 5A). Importantly, TIQ-A at 10 μM proved to be neuroprotective also when added immediately or up to 30 min after the termination of OGD (Fig. 5B).

By means of a flow-cytometric assay we also investigated the effects of TIQ-A on poly(ADP-ribosyl)ation in neurons exposed to OGD. As shown in Fig. 6, the neuronal content of PAR, the product of PARP-1 activity, was dramatically increased 1 h after OGD. This effect was almost completely prevented by the addition of 10 μM TIQ-A to the incubation medium.

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Discussion

PARP-1 is a 113 kDa dimeric protein characterized by three major domains: an N-terminal DNA-binding domain containing the nuclear localization sequence, an internal automodification region bearing a BRCA1 C-terminal domain, and a C-terminal which includes the catalytic domain (D'Amours et al., 1999; Smith, 2001). Although this complex structural organization might be used to develop enzymatic inhibitors targeting different functional domains, all the known chemical inhibitors of PARP-1 have been designed as substrate analogs. Among these, 3,4-dihydro-isoquinolinone derivatives are potent inhibitors that have been extensively investigated in *in vitro* (Suto et al., 1991; Arundel-Suto et al., 1991; Banasik et al., 1992; Zhang et al., 1994) and *in vivo* (Takahashi et al., 1997; Takahashi and Greenberg, 1999) models.

In the present study, we examined the pharmacological profile of new 3,4-dihydroisoquinolinone derivatives that were structurally designed as potential PARP-1 inhibitors and correlated their inhibitory activity with the neuroprotective effects on cultured cortical cells exposed to OGD. The IC_{50} values observed for 5OH-DIQ, DPQ and PND were somewhat higher than those previously reported (Suto et al., 1991; Banasik et al., 1992). Although comparison of IC_{50} values from different laboratories must be undertaken with caution, it should be noted that this is the first study comparing the inhibitory potencies of these three molecules using a commercially-available pure bovine PARP-1 preparation.

The evaluation of our novel compounds extends the existing information on the structure-activity relationship of PARP-1 inhibitors. In particular, the need of an *anti* disposition of the amido moiety of the 3,4-dihydro-isoquinolinone derivatives involved in a hydrogen bonding with the amido counterpart of the enzyme was confirmed by the lack of activity of compounds 4 and 5, endowed with a constrained *syn* disposition of the amido group. The weak inhibitory activity of the seven-membered derivative (compound 6) may

tentatively be ascribed to a shift in the orientation of the amido group which prevented the formation of a productive hydrogen bonding with the Gly863 site of the enzyme. We also investigated the effect of changing the substituent in the 5-position of dihydro-isoquinolin-1(2H)-one. Whereas the 5-hydroxy derivative (5OH-DIQ) was confirmed to have PARP inhibitory activity (Arundel-Suto et al., 1991), substitution with both the larger thiol or the benzoyloxy groups (as in compounds 1 and 2) caused a marked reduction in the potency, thus delineating a size-limited binding pocket. The more directional and electron rich acetylene group (compound 3) on the other hand, showed a potency similar to 5OH-DIQ.

The results obtained with DPQ derivatives showed that compounds 7 and 8, prepared with triple bonds in the side chain in order to constrain its binding orientation, were less potent than DPQ itself, suggesting that these changes do not favor their insertion in the enzyme active site. These results, however, confirmed the hypothesis that the hindrance of the side chain orientation affected DPQ potency, thus leaving open the possibility that different arrangements of the side chain group might allow us to obtain DPQ derivatives with increased inhibitory activity. Studies on compound 9 confirmed that a seven-membered ring inserted in the DPQ moiety causes a marked reduction in the inhibitory potency on PARP-1. Furthermore, the chimera made by combining the buthoxy-piperidine chain of DPQ with a quinazolinone moiety, a structure previously reported to be endowed with PARP-1 inhibitory activity (Griffin et al., 1998), led to compound 10, an inhibitor with an IC_{50} comparable to that of DPQ. Much more intriguing were the results obtained with the isosteric replacement of the PND benzene ring with a thiophene, which led to TIQ-A and to compounds 11 and 12, which were all potent PARP-1 inhibitors.

Because PARP-1 inhibitors are neuroprotective in experimental models of ischemic brain injury (Eliasson et al., 1997; Ha and Snyder, 2000; Moroni et al., 2001) we studied the effects of the new compounds on neuronal death after OGD in vitro. Our results show that

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selected PARP-1 inhibitors effectively attenuated OGD neurotoxicity and that the degree of neuroprotection correlated with their inhibitory potency on PARP-1 activity. TIQ-A and compound 11 were significantly more potent than DPQ in inhibiting both PARP-1 activity and OGD-induced neuronal death. Their IC_{50}s against OGD injury were: 0.15 ± 0.01 μM for TIQ-A, 0.20 ± 0.05 μM for compound 11, and 4.0 ± 0.8 μM for DPQ. Considering the need for drugs able to exert neuroprotection when given after the onset of cerebral ischemia in the clinical setting, the finding that TIQ-A is active in a post-treatment paradigm supports its potential therapeutic relevance.

The correlation between the neuroprotective properties and the potency in inhibiting PARP-1 activity of these compounds, along with the evidence that the formation of PAR is triggered by OGD and efficiently suppressed by TIQ-A, confirms the pivotal role of this enzyme in the pathophysiology of ischemic brain damage and underscores the therapeutic significance of these experimental compounds. Unfortunately, the rate of discovery of new PARP family members has exceeded that of PARP subtype-specific inhibitors. Therefore, we cannot rule out that the neuroprotective effects of the compounds reported here may be due to inhibition of family members other than PARP-1. Indeed, a very recent study demonstrates that, akin to PARP-1 inhibition, suppression of PARP-2 activity exerts anti-inflammatory effects (Popoff et al., 2002). However, whether PARP-2 and/or other PARP family members may participate in the pathogenesis of ischemic neuronal death is yet to be determined. In this scenario, the forthcoming development of PARP subtype-specific inhibitors (Perkins et al., 2001) will undoubtedly help to elucidate the role of the various members of the family in physiology and pathology.

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Footnotes

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Address reprints requests to: Prof. Flavio Moroni, Dipartimento di Farmacologia Preclinica e Clinica, Università di Firenze, Viale Pieraccini, 6, 50139 Firenze, Italy

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Figure legends

Figure 1. Molecular structures of the known PARP-1 inhibitors benzamide, 3,4-dihydro-5-hydroxy-1(2H)-isoquinolinone (5OH-DIQ), 3,4-dihydro-5-[4-(1-piperidinyl)buthoxyl)]-1(2H)-isoquinolinone (DPQ), and 6(5H)-phenantridinone (PND).

Figure 2. Molecular structures of newly synthesized isoquinolinone, DPQ and PND derivatives.

Figure 3. Antagonist activity of isoquinolinone, DPQ and PND derivatives on PARP-1 activity *in vitro*. Values are expressed as dpm/h and were obtained by incubating the inhibitor with 0.2 μCi [adenine-2,8-^{3}H]NAD and 0.13 U partially purified bovine PARP-1 for 1 h at 37°C. A, isoquinolinone derivatives with no switch into the *syn* conformation of the amide group of nicotinamide (compounds 1, 2 and 3) are effective in inhibiting PARP-1 activity in the μM range. B, DPQ derivatives such as compound 7, 8 and 10 are effective in reducing PARP-1 activity. C, PND derivatives with a thiophene group (TIQ-A, compounds 11 and 12) are very potent PARP-1 inhibitors, with IC_{50}s between 1-10 μM. Each point represents the mean ± S.E.M. of at least 3 experiments performed in triplicate.

Figure 4. Neuroprotection by isoquinolinone, DPQ and PND derivatives against OGD neurotoxicity in murine cortical cultures and correlation with their PARP-1 inhibitory potency. A, selected PARP-1 inhibitors reduce OGD-induced neuronal death. OGD was applied for 60 min and 24 h later neuronal damage was assessed by measuring the release of LDH in the medium. Each PARP-1 inhibitor was added at a concentration of 100 μM during OGD and the subsequent 24 h recovery period. Data are expressed as percent of glutamate-

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induced neuronal damage. Each bar represents the mean ± S.E.M. of at least 5 experiments. *, $p < 0.05$ and **, $p < 0.01$ versus OGD (ANOVA + Tukey's w test). B, positive correlation ($P<0.001$) between the IC_{50} values of selected compounds for the inhibition of PARP-1 activity and their IC_{50} values for the reduction of OGD-induced LDH release. Pearson's r correlation coefficient was calculated between the two IC_{50} values, correlation was verified by non-parametric (Spearman's) rank correlation and the *p* value was calculated using a two-tailed test.

Figure 5. PND, TIQ-A and compound 11 protect against OGD neurotoxicity in murine cortical cultures. A, Neuronal death was significantly reduced by addition of 0.1-100 µM TIQ-A or compound 11 and of 10-100 µM PND to the incubation medium during OGD exposure and the subsequent 24 h recovery period. Each point represents the mean ± S.E.M. of at least five experiments. B, TIQ-A was neuroprotective also when added during or at 0, 15 and 30 min after OGD. Each point represents the mean ± S.E.M. of at least four experiments. *, $p < 0.05$ and **, $p < 0.05$ versus OGD alone.

Figure 6. TIQ-A (10µM) reduces the early formation of PAR induced by 60 min OGD in cortical neurons. One hour after OGD, neuronal cultures were fixed, labeled with antibodies directed against PAR and GFAP, and processed for flow-cytometry analysis. The GFAP-positive contribution to PAR fluorescence was subtracted from all samples. The graph plots the relative number of cells displaying increasing levels of PAR fluorescence and is representative of four analyses.

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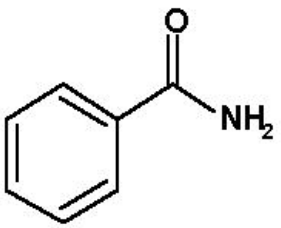
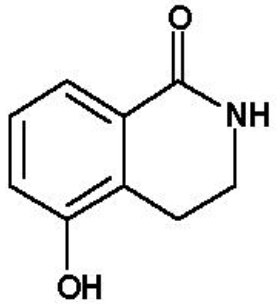
Table 1. Antagonist activity of various isoquinolinone, DPQ and PND derivatives upon PARP-1 activity *in vitro*.

IC_{50} values (mean ± s.e. mean), i.e. the concentrations of half-maximal inhibition of *in vitro* PARP-1 activity, were calculated from the curves shown in Figure 3 by nonlinear regression analysis using the GaphPad Prism software package (San Diego, CA).

Isoquinolinone derivatives		**DPQ derivatives**		**PND derivatives**	
Compound	IC_{50} (μM)	Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
5OH-DIQ	**17 ± 4**	**DPQ**	**2.2 ± 0.5**	**PND**	**3.4 ± 0.7**
1	1000 ± 150	**7**	34 ± 4	**TIQ-A**	0.45 ± 0.10
2	251 ± 45	**8**	8 ± 2	**11**	0.39 ± 0.19
3	13 ± 0.4	**9**	> 100	**12**	0.21 ± 0.10
4	> 100	**10**	3.4 ± 0.3		
5	> 100				
6	130 ± 6				



Benzamide



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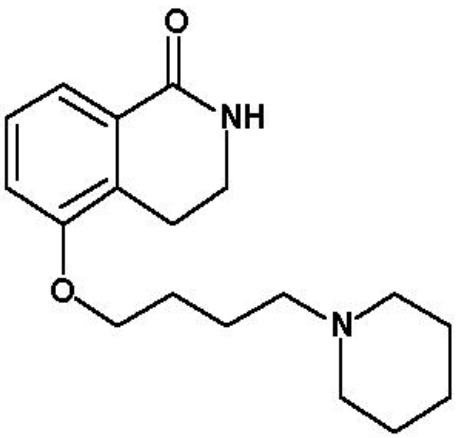
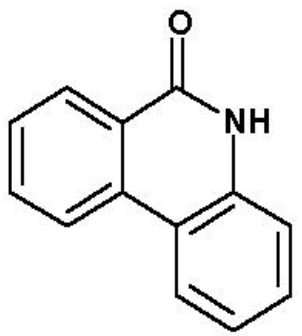


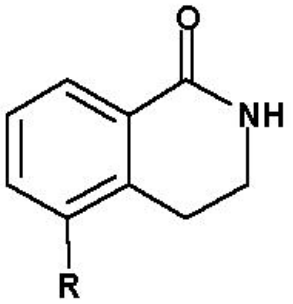
DPQ



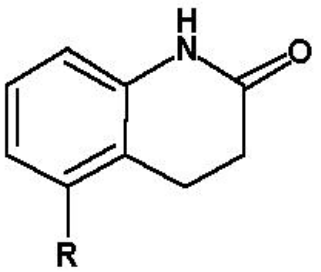
PND

Chiarugi_fig.1

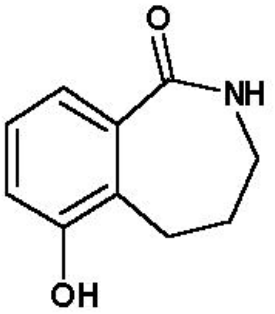
A) Body modification on isoquinolinone derivatives



R_1 = SH *(1)*
R_2 = OCOPh *(2)*
R_3 = C≡CH *(3)*

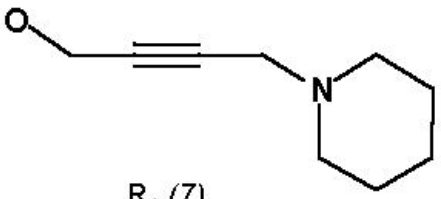


R_1 = OH *(4)*
R_2 = OCOPh *(5)*

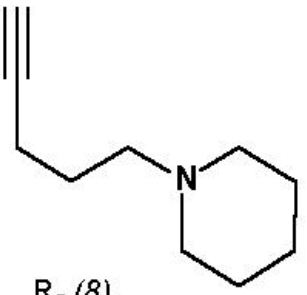


(6)

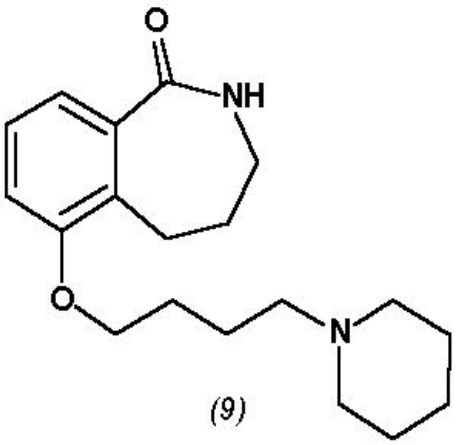
B) DPQ-like compounds



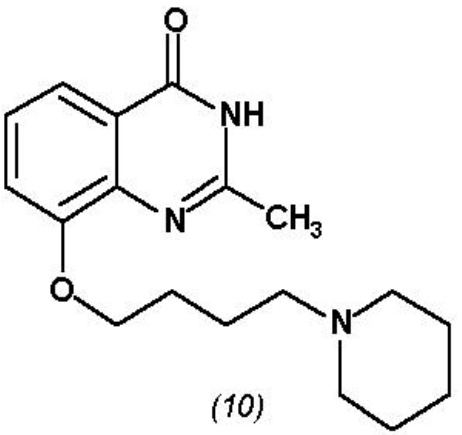
R_4 *(7)*



R_5 *(8)*

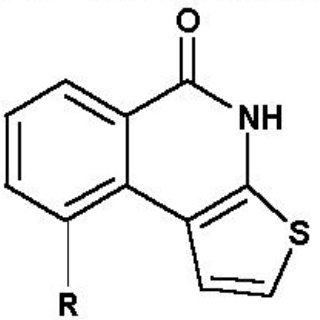


(9)



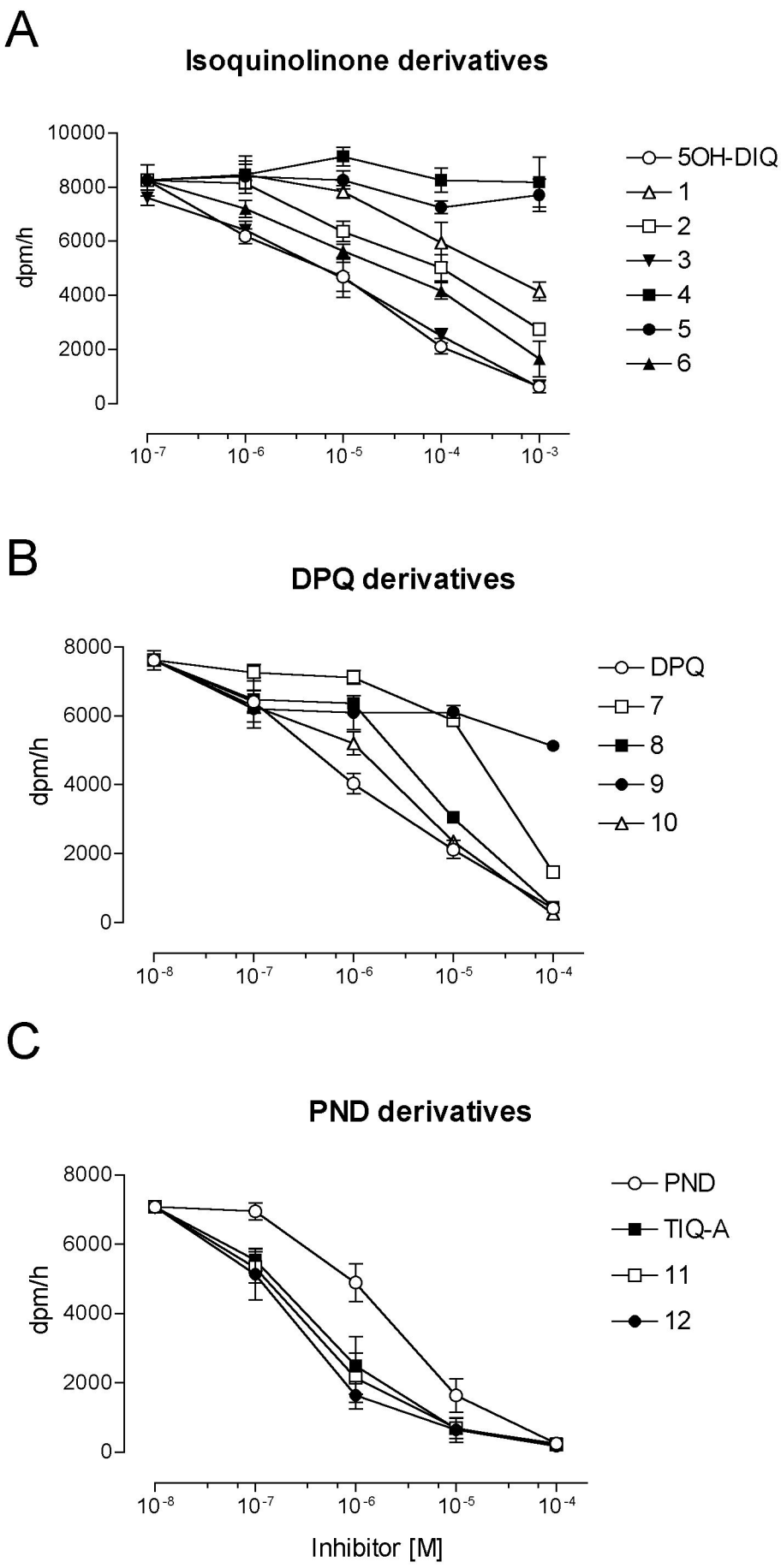
(10)

C) PND-like compounds

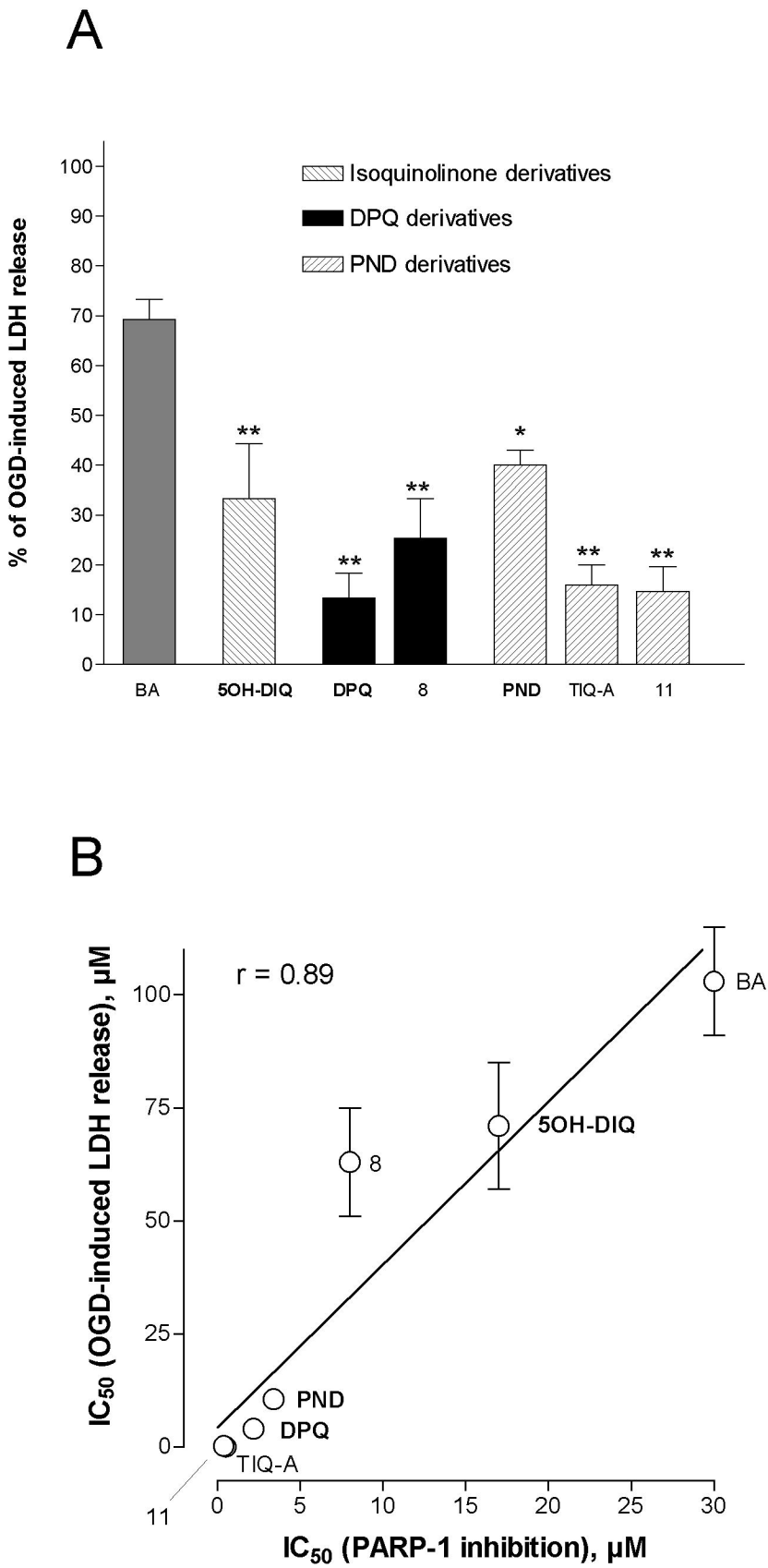


R_1 = H *(TIQ-A)*
R_2 = OH *(11)*
R_3 = OCH_3 *(12)*

Chiarugi_fig.2



Chiarugi_fig. 3



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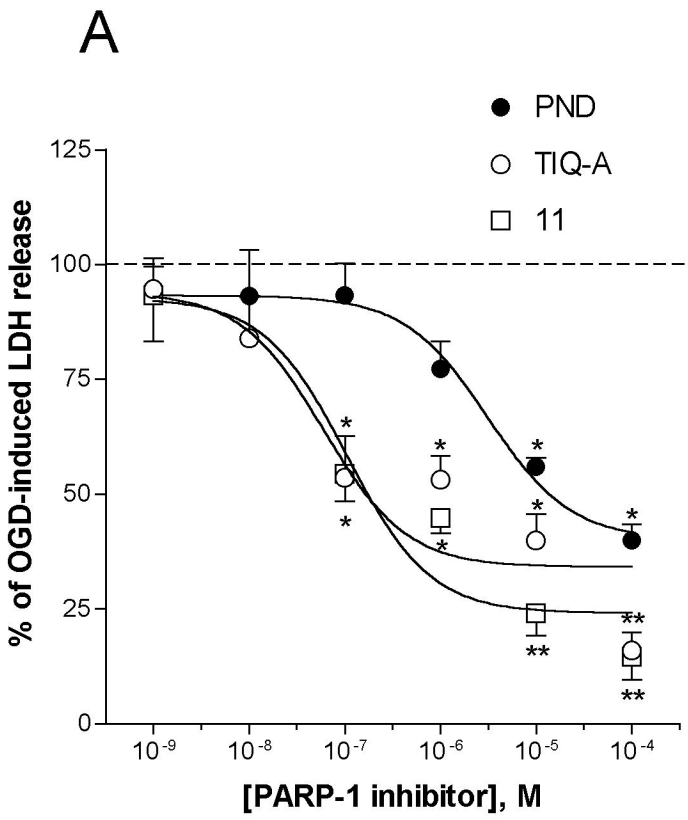
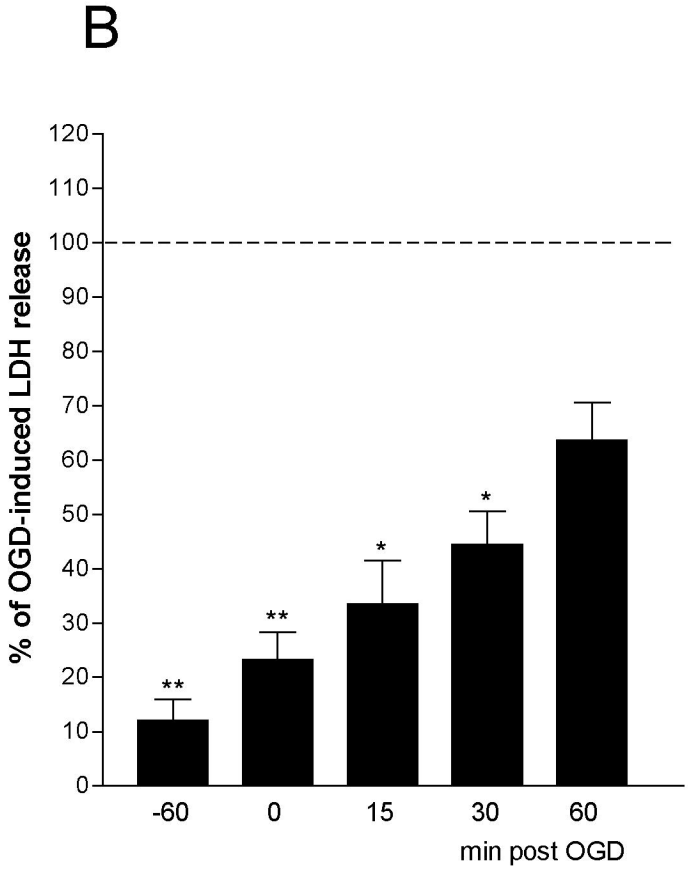
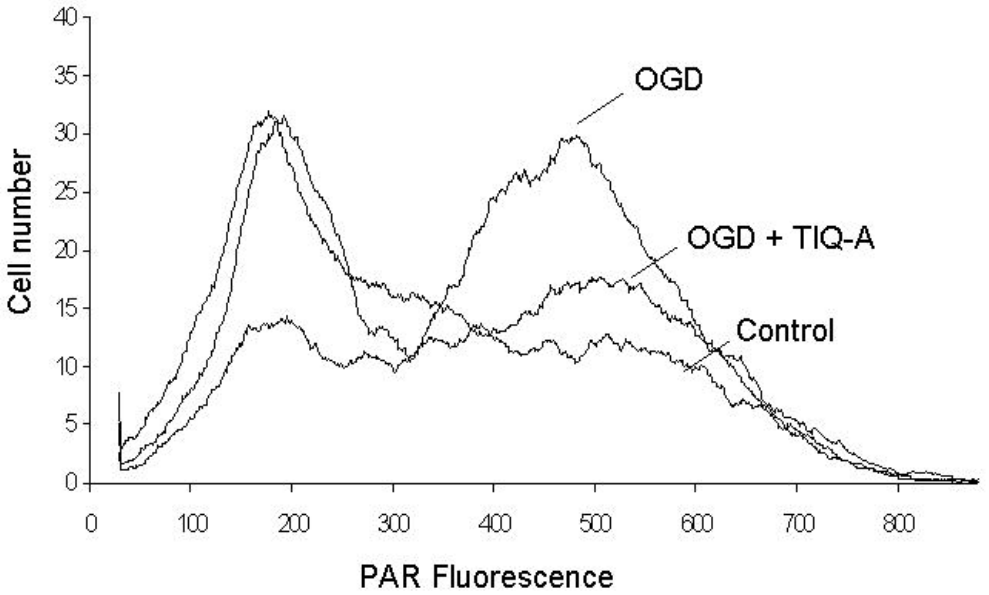




Chiarugi_fig. 5


Cell number
PAR Fluorescence
OGD
OGD + TIQ-A
Control
0
5
10
15
20
25
30
35
40
0
100
200
300
400
500
600
700
800