

Role of poly(ADP-ribose) synthetase in inflammation and ischaemia-reperfusion

Csaba Szabó and Valina L. Dawson

Oxidative and nitrosative stress can trigger DNA strand breakage, which then activates the nuclear enzyme poly(ADP-ribose) synthetase (PARS). This enzyme has also been termed poly(ADP-ribose) polymerase (PARP) or poly(ADP-ribose) transferase (pADPRT). Rapid activation of the enzyme depletes the intracellular concentration of its substrate, nicotinamide adenine dinucleotide, thus slowing the rate of glycolysis, electron transport and subsequently ATP formation. This process can result in cell dysfunction and cell death. In this article, **Csaba Szabó and Valina Dawson** overview the impact of pharmacological inhibition or genetic inactivation of PARS on the course of oxidant-induced cell death *in vitro*, and in inflammation and reperfusion injury *in vivo*. A major trigger for DNA damage in pathophysiological conditions is peroxynitrite, a cytotoxic oxidant formed by the reaction between the free radicals nitric oxide and superoxide. The pharmacological inhibition of poly(ADP-ribose) synthetase is a novel approach for the experimental therapy of various forms of inflammation and shock, stroke, myocardial and intestinal ischaemia-reperfusion, and diabetes mellitus.

Poly(ADP-ribose) synthetase (PARS) [EC 2.4.2.30, also called poly(ADP-ribose) polymerase (PARP) or poly(ADP-ribose) transferase (pADPRT)] is a protein-modifying and nucleotide-polymerizing enzyme that is present abundantly in the nucleus¹⁻³. On average, approximately one molecule of this enzyme is present per 1000 base pairs of DNA. PARS consists of the DNA-binding amino-terminal domain (42 kDa), the central automodification domain (16 kDa) and the carboxy-terminal catalytic domain (55 kDa). The DNA-binding domain uses two zinc fingers that recognize single- or double-strand breaks in double-stranded DNA. The central, highly conserved domain is autoADP-ribosylated by PARS, whereas the catalytic domain is involved in the synthesis of the poly(ADP-ribose) polymer¹⁻³.

The obligatory trigger of PARS activation is nicks and breaks in the DNA strand, which can be induced by a

variety of environmental stimuli and free radical (or oxidant) attacks; these include the oxidants hydrogen peroxide, hydroxyl radical and peroxynitrite, ionizing radiation, and genotoxic agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). In response to DNA damage, PARS becomes activated and, using nicotinamide adenine dinucleotide (NAD⁺) as a substrate, it builds up homopolymers of adenosine diphosphate ribose units. Poly(ADP-ribose) acceptors include histones, topoisomerases I and II, DNA polymerases and DNA ligase 2, as well as PARS itself. PolyADP-ribosylation might result in the inhibition of the activity of some of these enzymes. In the case of histones, polyADP-ribosylation stimulates chromatin relaxation. Poly(ADP-ribose) catabolism and metabolism is a dynamic process, with poly(ADP-ribose) glycohydrolase playing the major role in the degradation of the polymer¹⁻³.

The physiological function of PARS and poly(ADP-ribosylation) is still under heavy debate. From studies using pharmacological inhibitors of PARS, poly(ADP-ribosylation) has been suggested to regulate gene expression and gene amplification, cellular differentiation and malignant transformation, cellular division and DNA replication, as well as apoptotic cell death¹⁻⁶. However, recent studies using cells from PARS(-/-) mice have failed to demonstrate a role for PARS in the process of apoptosis induced by various apoptotic signals, such as the Fas ligand or dexamethasone⁶⁻⁹. The proposed role of PARS in DNA repair^{4,5} has not yet been fully explored, as cells from PARS(-/-) mice can display both normal⁶ and impaired⁹ DNA repair, depending on the nature of the stimulus of DNA injury. It appears that PARS, along with DNA-dependent protein kinase, plays an important role in maintaining genomic stability^{6,8}. The clarification of the physiological role of PARS requires further studies using selective PARS inhibitors and modern molecular biological approaches.

The suicide theory of PARS activation

The cellular NAD⁺ concentration is known to regulate an array of vital cellular processes. NAD⁺ serves as a cofactor for glycolysis and the tricarboxylic acid cycle, thus providing ATP for most cellular processes. NAD⁺ also serves as the precursor for nicotinamide adenine dinucleotide phosphate (NADP), which acts as a cofactor for the pentose shunt, for bioreductive synthetic pathways, and is involved in the maintenance of reduced glutathione pools. The observation that the activation of PARS can lead to massive NAD⁺ usage that correlates with changes in the cellular amount of NAD⁺ led Berger and Okamoto to propose that consumption of NAD⁺ due to DNA damage and activation of PARS can affect cellular energetics and function^{10,11}. In the 1980s, many investigators observed rapid depletion of NAD⁺ due to PARS activation, leading to cellular ATP depletion and functional alterations of the cell, with eventual necrotic-type cell death. The main cytotoxic triggers used in these studies *in vitro* were alkylating agents, radiation and

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Table 1. Protection against cellular injury by poly(ADP-ribose) synthetase inhibition *in vitro*

Target cell	Inducer of injury	Mode of PARS inhibition	Effect of PARS inhibition	Refs
Murine islet cells	Streptozotocin Alloxan	3-AB Nicotinamide	Protection against islet cell injury and against the inhibition of proinsulin synthesis	12, 13
L1210 murine leukaemia cells L929 fibroblasts	MNNG	3-AB Nicotinamide	Prevention of the suppression of NAD and ATP levels and the changes in cellular glucose-6-phosphate levels	14, 15
Murine macrophages Human lymphocytes	Hydrogen peroxide	3-AB Nicotinamide	Prevention of NAD loss, ATP loss and cell death; no effect on the hexose monophosphate shunt	16, 17
Human umbilical vein endothelial cells	XO/hypoxanthine Glucose oxidase/glucose	3-AB	Inhibition of NAD loss; no effect on ATP depletion	18
Human ovarian and breast tumour lines Murine L929 fibroblasts	TNF- α	3-AB Nicotinamide	Protection against cytotoxicity in the L9292 cells; enhancement of cytotoxicity in the oncogene-expressing lines; no effect in non-expressing human cells	19, 20
Bovine pulmonary or cardiac endothelial cells	Hydrogen peroxide Dihydroxyfumarate Menadione	3-AB Nicotinamide	No effect on DNA strand breaks, but prevention of NAD and ATP loss and reduction in LDH release	21, 22
Mastocytoma and lymphoma cell lines Fibroblasts	Cytotoxic T-cell attack	3-AB Benzamide PARS(-/-) cell line	Reduced target cell lysis	23, 24
Human T cells	γ -Irradiation	3-AB Nicotinamide	Protection against the loss of viability and restoration of the impaired T-cell adhesion to endothelial cells	25
Human lymphocytes	Hydrogen peroxide γ -Irradiation XO/hypoxanthine	3-AB Nicotinamide	Preservation of GSH and reduced thiol content (NAD depletion was not associated with ATP depletion)	26
Murine L929 fibroblasts Rat primary hepatocytes	<i>Tert</i> -butyl-hydroperoxide	3-AB Benzamide	Fibroblasts, but not hepatocytes, are protected against the metabolic changes	27
Human endothelial cells	XO/hypoxanthine	3-AB	Prevention of the ATP depletion, improved cellular viability	28
Rat hippocampal slices	Hypoxic injury Fluid percussion	3-AB Nicotinamide	Improvement of orthodromic and antidromic population spike recovery	29, 30
Murine pancreatic islet cells	Nitroprusside SNAP XO/hypoxanthine	3-AB Nicotinamide 4-Amino-1,8-naphthalimide PARS(-/-) phenotype	Prevention of NAD depletion and islet cell lysis	31–33
Rat cerebellar granule cells	Glutamate	3-AB Benzamide 3-Aminophthalhydrazide	Protection against neuronal death, without affecting glutamate-induced Ca ²⁺ influx	34

hydrogen peroxide, whereas the most frequently used PARS inhibitors were nicotinamide, 3-aminobenzamide and benzamide (Table 1; Refs 12–51).

Research into the 'suicidal' role of PARS gained new momentum in the mid-1990s because of the observations *in vitro* that NO, a reactive free radical species produced from L-arginine by a family of enzymes called NO synthases (NOS), or peroxynitrite, a highly reactive oxidant species produced from NO and superoxide, can trigger DNA single-strand breakage and PARS activation^{32,40,44}. NO and peroxynitrite can also inhibit mitochondrial respiration and exert other cytotoxic effects on their own. It

is likely that a synergistic relationship exists between the PARS-mediated pathways and PARS-independent pathways of cellular metabolic suppression.

The observations that NO and peroxynitrite are important mediators of the cellular damage in various forms of inflammation and reperfusion injury suggested that the PARS-related suicide pathway might play a role in various pathophysiological conditions *in vivo*. The research into the role of PARS in these conditions has also been enhanced significantly by new molecular biology tools, such as the development of novel, more potent inhibitors of PARS and the generation of

Table 1. contd

Target cell	Inducer of injury	Mode of PARS inhibition	Effect of PARS inhibition	Refs
Rat cardiomyocytes; cardiac myoblast cell line	Hydrogen peroxide Peroxynitrite	3-AB Nicotinamide	Protection against the loss of NAD and ATP; maintained cardiac action potentials; inhibition of peroxidation	35, 36
Pulmonary fibroblasts	Peroxynitrite Hypoxia/reoxygenation	PARS(-/-) phenotype	Prevention of cell death	36, 37
Murine lung slices	Cyclophosphamide	3-AB	Protection against NAD loss and LDH release	38
Cerebral cortical culture	NMDA Sodium nitroprusside SNAP SIN-1	Benzamide 3-AB 4-AB 1,5-dihydro- isoquinoline PARS(-/-) phenotype	Prevention of cell death	39, 40
Rabbit type II pneumocytes	Hydrogen peroxide	3-AB Nicotinamide	Maintenance of NAD and ATP levels and surfactant synthesis	41
Intestinal epithelial cell line HT-29-18-C1	Hydrogen peroxide	3-AB Nicotinamide	Prevention of the loss of NAD and ATP, and protection against cell death; no effect on cell detachment	42
Human lymphocytes	Mustard nitrogen	Nicotinamide 3-AB	Protection against cell death, both in pre-treatment and post-treatment regimens	43
Murine macrophages Rat aortic smooth muscle cells Endothelial cells	Peroxynitrite LPS/IFN	3-AB Nicotinamide INH2BP	Prevention of NAD and ATP depletion and suppression of mitochondrial respiration; maintenance of endothelial and smooth muscle functions	37, 44–46
Perfused rat lungs	NMDA	3-AB 6(5H)phenanth- ridinone	Protection against lung oedema formation and protein extravasation	47
YAC-1 lymphoma cells	SNAP SNP Hydrogen peroxide	3-AB Nicotinamide	Protection against cell lysis	48
Human neuroblastoma line SH-SY5Y	SNP SNAP	3-AB 1,5-Isoquino- linediol	Protection against cell death	49
Human pulmonary and intestinal epithelial cells	Peroxynitrite SIN-1	3-AB INH2BP	Protection against NAD and ATP depletion; amelioration of epithelial hyperpermeability	50, 51

/, in combination with; AB, aminobenzamide; GSH, reduced glutathione; IFN- γ , interferon γ ; INH2BP, 5-iodo-6-amino-1,2-benzopyrone; LDH, lactate dehydrogenase; LPS, bacterial lipopolysaccharide; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; NMDA, *N*-methyl-D-aspartate; PARS, poly(ADP-ribose) synthetase; SIN-1, 3-morpholino-sydnominine; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; SNP, sodium nitroprusside; TNF- α , tumour necrosis factor α ; XO, xanthine oxidase.

genetically engineered animals lacking the gene encoding PARS. From these recent studies, PARS emerges as one of the key final mediators of cellular injury in a variety of pathophysiological conditions (Table 2, Refs 52–70).

Role of PARS in inflammation

The earliest implication of PARS in pathophysiology was related to pancreatic islet cell injury and diabetes. Yamamoto and co-workers demonstrated that in streptozotocin-treated islet cells, inhibition of PARS with 3-aminobenzamide prevented NAD⁺ depletion and the suppression of pro-insulin synthesis without modifying

the extent of DNA damage¹¹. Subsequent experiments *in vivo* demonstrated that inhibition of PARS by 3-aminobenzamide or nicotinamide delays the onset of streptozotocin- and alloxan-induced diabetes (Table 2). The above observations can now be re-evaluated based on the current evidence demonstrating that (1) streptozotocin generates NO in aqueous solutions⁷¹, and (2) NO scavengers protect against streptozotocin-induced DNA strand breakage⁷². Based on these observations, it is logical to propose that DNA single-strand breakage due to NO and peroxynitrite generation underlies the pathogenesis of PARS activation and subsequent islet cell damage in response to streptozotocin. Indeed, islet cells

Table 2. Protection against cellular injury by poly(ADP-ribose) synthetase inhibition *in vivo*

Disease model	Mode of PARS inhibition	Effect of PARS inhibition	Refs
Diabetes (streptozotocin- and alloxan-induced, or obesity associated) in rodents	3-AB Nicotinamide	Increased plasma and pancreatic insulin concentration; reduced hyperglycaemia	52–54
Adjuvant or collagen arthritis in rodents	3-AB Nicotinamide	Reduced degree of arthritis	55–57
Liver damage induced by acetaminophen or 1,2-dibromo-3-chloropropane in rodents	Nicotinamide	Reduced organ injury	58, 59
Rat endotoxic shock	3-AB INH2BP Nicotinamide	Improved vascular function and haemodynamic status; maintained cellular NAD and ATP in peritoneal macrophages; improved survival rate	37, 46, 60
Metamphetamine- or MPTP-induced neurotoxicity in mice	Benzamide 3-AB 4-AB Nicotinamide 1,5-Dihydroisoquinoline	Reduced degree of striatal dopamine depletion	61, 62
Transient focal ischaemia–reperfusion rodents	3-AB 3,4-Dihydro-5-[4-1(1-piperidynil butoxy)-1(2H)-isoquinolinone	Suppression in infarct size	63, 64
Transient focal, cerebral ischaemia–reperfusion in mice	PARS(–/–) phenotype	Marked suppression in infarct size	40, 63
Rat and mouse zymosan- and carrageenan-induced multiple organ failure	3-AB PARS(–/–) phenotype	Reduction in oedema formation, lung and liver injury, and suppression of neutrophil recruitment into the inflamed organs; reduction in the apparent reactive peroxynitrite formation	65, 66
Rat splanchnic ischaemia–reperfusion	3-AB	Reduction in epithelial hyperpermeability, improved histology, reduced neutrophil infiltration; reduction in the apparent reactive peroxynitrite formation	67
Rat and rabbit myocardial ischaemia–reperfusion	3-AB 4-Amino-1,8-naphthalimide 4-AB; 1,5-Dihydroisoquinoline	Reduction in infarct size; improved myocardial contractility; reduced plasma creatinine phosphokinase levels; improved myocardial ATP; reduced neutrophil infiltration; reduction in the apparent reactive peroxynitrite formation	68, 69
Skeletal muscle ischaemia–reperfusion in the rabbit	3-AB Nicotinamide 1,5-Dihydroisoquinoline	Reduction in the degree of muscle necrosis	69
Rat model of retinal ischaemia–reperfusion	3-AB	Prevention of loss of inner retinal thickness; improved histology	70

AB, aminobenzamide; INH2BP, 5-iodo-6-amino-1,2-benzopyrone; PARS, poly(ADP-ribose) synthetase.

from PARS(–/–) knockout mice are resistant to injury in response to NO generator and oxyradical generator compounds, compared with islet cells from wild-type animals³³. However, under extreme oxidant stress, an overwhelming, PARS-independent, cytotoxicity takes over the role of the PARS-related pathway of cellular injury³³. Although the evidence regarding the effectiveness of PARS inhibitors against oxidant-induced islet cell injury is solid, the relevance of these findings in relation to spontaneous, autoimmune diabetes remains unclear. Recently, it has been shown that the PARS inhibitor nicotinamide reduces the degree of obesity-associated diabetes⁵⁴. In other models of spontaneous diabetes, such as in autoimmune diabetes-prone, non-obese, diabetic mice, the expression of the inducible

(macrophage-type, immunological) isoform of NOS (iNOS) and the formation of peroxynitrite has been demonstrated^{73–75}. However, clarification of the potential role of PARS activation in auto-immune islet damage requires further investigation.

More recent studies have implicated PARS in a variety of other local or systemic inflammatory conditions. In carrageenan- or zymosan-induced paw oedema and pleurisy models, which are associated with the expression of iNOS and the production of peroxynitrite (Fig. 1), inhibition of PARS by 3-aminobenzamide reduces oedema formation, mononuclear cell infiltration and tissue injury^{65,66}. PARS(–/–) mice are resistant to zymosan-induced multiple organ failure and neutrophil infiltration compared with wild-type littermates⁶⁵

(Fig. 2). Inhibition of PARS suppresses the development of rheumatoid arthritis in rodent models⁵⁵⁻⁵⁷.

Systemic inflammatory conditions, such as various forms of circulatory shock, are associated with the expression of iNOS and with the generation of peroxynitrite, resulting in a reduced responsiveness of blood vessels to vasoconstrictor agents (vascular hyporeactivity), myocardial dysfunction and impaired intracellular energetic processes, culminating in multiple organ failure⁷⁶. Experiments in rodents subjected to endotoxic shock have demonstrated that PARS activation plays a role in the pathogenesis of vascular failure and cellular energetic dysfunction^{37,45,46,76,77}. Importantly, inhibition of PARS, either with 3-aminobenzamide or with 5-iodo-6-amino-1,2-benzopyrone improves the survival rate of mice injected with a high dose of endotoxin^{46,77}.

Role of PARS in ischaemia-reperfusion

It is well established that oxygen-derived free radicals and oxidants play a role in the pathogenesis of ischaemia-reperfusion. Recent studies have also shown that superoxide, which is produced during the reperfusion phase, rapidly reacts with NO to form peroxynitrite⁸³⁻⁸⁵. Preventing the generation of oxyradicals or peroxynitrite by using superoxide-neutralizing strategies or selective inhibition of NOS protects against the development of organ injury⁷⁸⁻⁸⁰.

Neural damage following stroke is thought to be elicited in large part by a massive release of the excitatory neurotransmitter glutamate acting upon N-methyl-D-aspartate (NMDA) and other excitatory amino acid receptors^{81,82}. In many animal species, glutamate receptor antagonists block the neural damage that follows vascular stroke as well as the neurotoxicity elicited by treating cerebral cortical cultures with glutamate or NMDA (Ref. 83). Neurotoxicity elicited by the stimulation of NMDA receptors is mediated mainly by the formation of NO. NMDA neurotoxicity is diminished by NOS inhibitors^{84,85} and by targeted disruption of the neuronal isoform of NOS (nNOS)⁸⁶. Infarct volume following vascular stroke is also markedly diminished in animals treated with NOS inhibitors^{83,87} and specifically in mice in which the gene encoding NOS has been disrupted⁸⁸ (Fig. 3).

Ischaemia-reperfusion can be modelled *in vitro* by exposing primary neuronal cultures to glutamate or its agonists, to NO donors, or by combined oxygen-glucose deprivation. In cerebellar granular cells exposed to toxic concentrations of glutamate, Cosi and colleagues demonstrated directly an increase in the content of cellular poly(ADP-ribose)⁸⁹. PARS inhibitors are neuroprotective in models of neuronal injury *in vitro* that are induced by glutamate or NO generators^{39,94}. The rank order of potency of different classes of PARS inhibitors correlates with the rank order of the degree of neuroprotection^{39,40}. Moreover, the protection by PARS inhibition is not associated with changes in glutamate-induced Ca²⁺ influx⁸⁹. In a more recent study, primary cortical

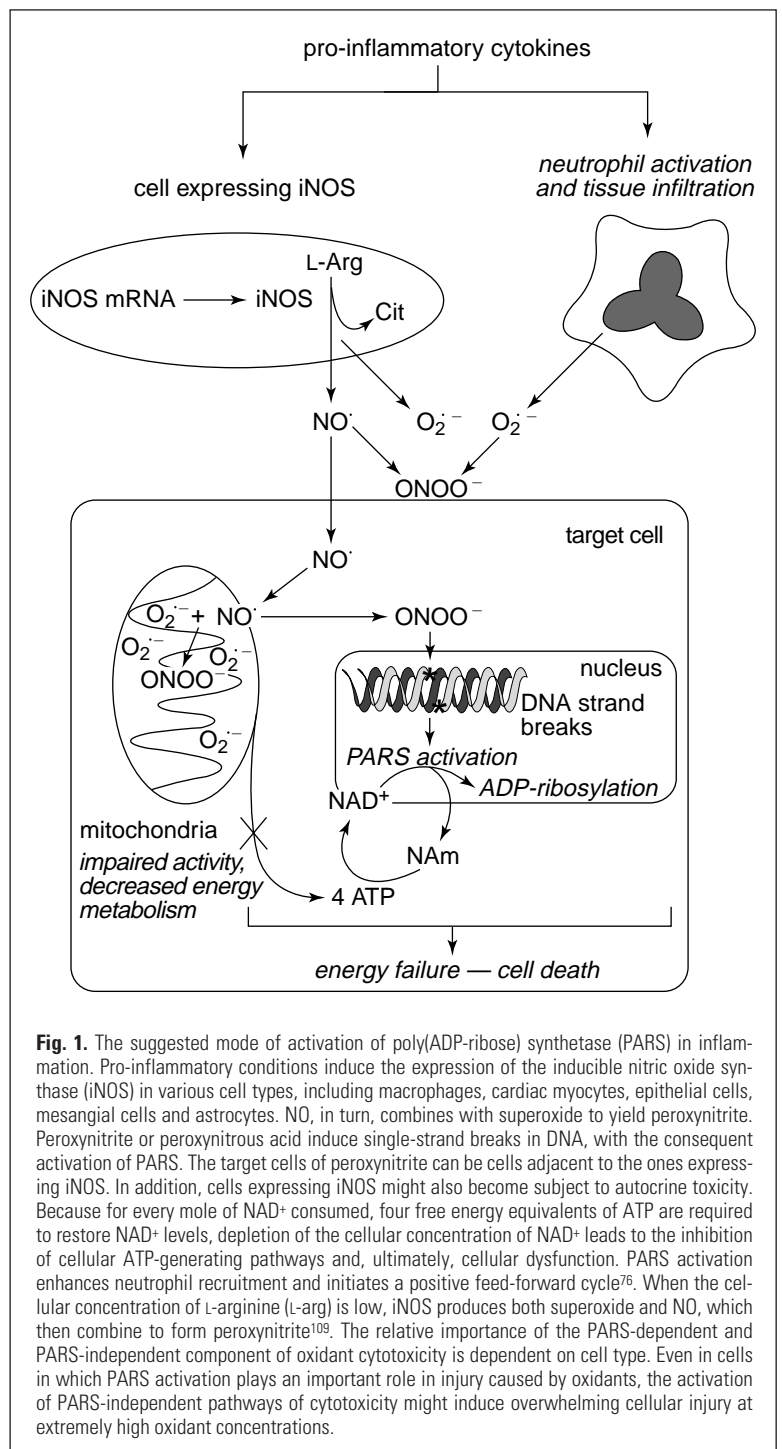
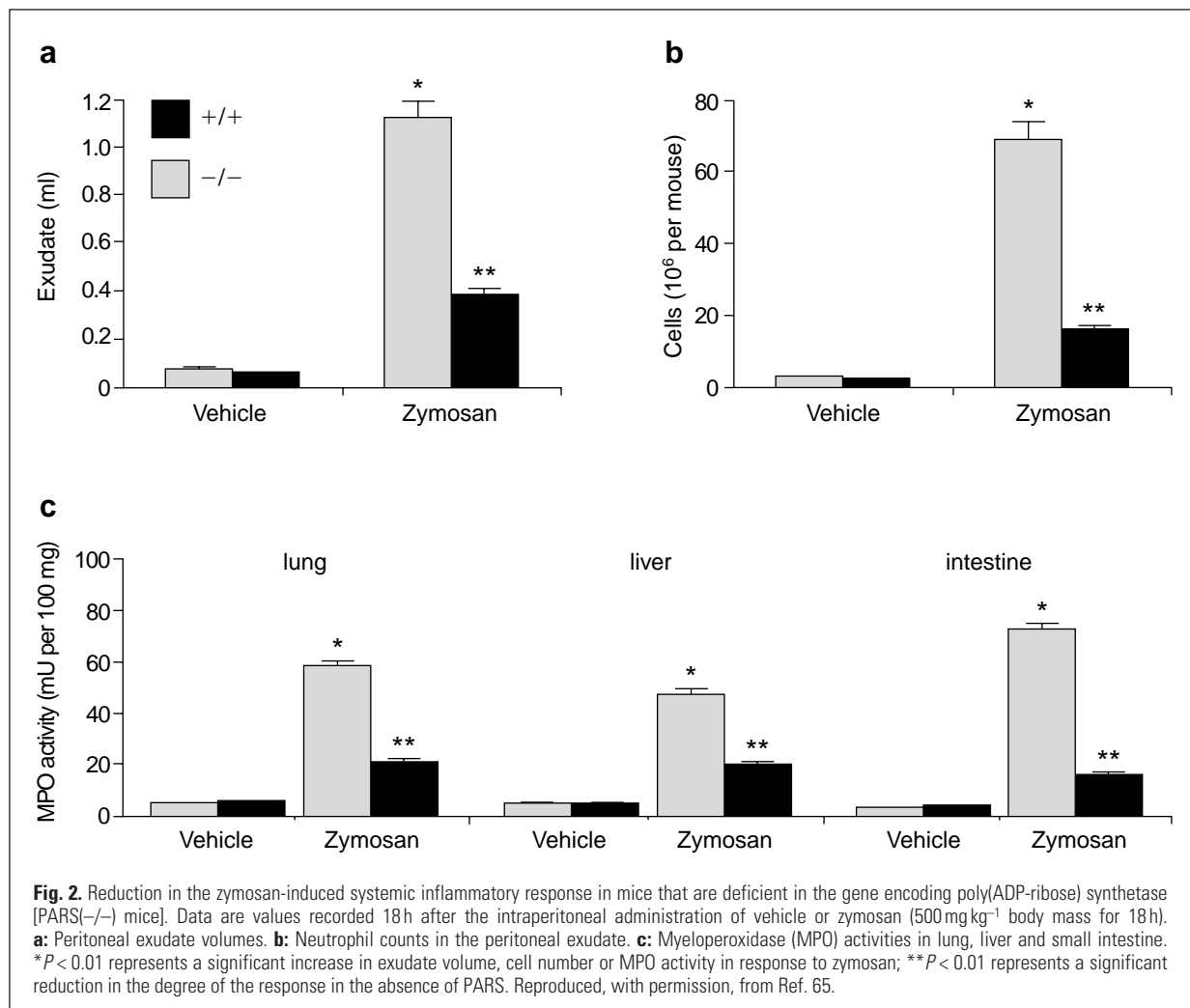


Fig. 1. The suggested mode of activation of poly(ADP-ribose) synthetase (PARS) in inflammation. Pro-inflammatory conditions induce the expression of the inducible nitric oxide synthase (iNOS) in various cell types, including macrophages, cardiac myocytes, epithelial cells, mesangial cells and astrocytes. NO, in turn, combines with superoxide to yield peroxynitrite. Peroxynitrite or peroxynitrous acid induce single-strand breaks in DNA, with the consequent activation of PARS. The target cells of peroxynitrite can be cells adjacent to the ones expressing iNOS. In addition, cells expressing iNOS might also become subject to autocrine toxicity. Because for every mole of NAD⁺ consumed, four free energy equivalents of ATP are required to restore NAD⁺ levels, depletion of the cellular concentration of NAD⁺ leads to the inhibition of cellular ATP-generating pathways and, ultimately, cellular dysfunction. PARS activation enhances neutrophil recruitment and initiates a positive feed-forward cycle⁷⁶. When the cellular concentration of L-arginine (L-arg) is low, iNOS produces both superoxide and NO, which then combine to form peroxynitrite¹⁰⁹. The relative importance of the PARS-dependent and PARS-independent component of oxidant cytotoxicity is dependent on cell type. Even in cells in which PARS activation plays an important role in injury caused by oxidants, the activation of PARS-independent pathways of cytotoxicity might induce overwhelming cellular injury at extremely high oxidant concentrations.

cultures from PARS(-/-) mice were found to be resistant to toxicity from NMDA, NO generators and combined oxygen-glucose deprivation⁴⁰.

The above-mentioned systems *in vitro* model only a component of the complex chain of events initiated *in vivo* following an ischaemic insult or stroke. Nevertheless, the pathophysiological relevance of observations *in vitro* is supported by the finding that in PARS(-/-) mice, a markedly reduced infarct volume occurs in a model of transient middle cerebral artery occlusion (Fig. 4)^{40,63}. The reduction in infarct volume was observed in



PARS(-/-) mice that had an identical genetic background to the wild-type strain⁴⁰. Because a mixed 129/C57B6 PARS(-/-) line also had reduced infarct volumes⁶³, the reduction in infarct volume was due to the absence of the PARS gene product and not to other genetic variables. PARS activation was examined following a focal ischaemic insult in the ipsilateral hemisphere by evaluating the formation of ADP-ribose polymer or levels of NAD⁺. ADP-ribose formation was increased and the concentration of NAD⁺ was decreased following a focal ischaemic insult in wild-type tissue; however, in PARS(-/-) tissue, no ADP-ribose formation was observed⁶³ and NAD⁺ levels were unaltered⁶³. It is believed that PARS activation is mainly related to NO production by nNOS because in nNOS-deficient mice subjected to middle cerebral artery occlusion-reperfusion, PARS activation is markedly diminished⁹⁰. A novel potent PARS inhibitor, 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2*H*)-isoquinolinone, also prevents neural damage following vascular stroke in rats⁶⁴. The profound neuroprotection observed in the PARS(-/-) mice exceeds the degree of protection reported for any other transgenic model, including the nNOS(-/-) mice. This observation suggests that PARS is activated by other

excitotoxic mechanisms, as well as by the production of free radicals and NO.

The activation of PARS might also be responsible, at least partly, for the development of reperfusion injury in the heart. In a rabbit model of myocardial infarction, pharmacological inhibitors of PARS, such as nicotinamide or 3-aminobenzamide, which are given immediately before the reperfusion of the ischaemic myocardium, dramatically reduce the infarct size; on the other hand, the structurally related, but inactive, agent 3-aminobenzoic acid is without protective effect⁶⁹. Similarly, in anaesthetized rats, 3-aminobenzamide exerts cardioprotective effects in a model of myocardial infarction elicited by occlusion and reperfusion of the left coronary artery⁶⁸; in this study, the following functional parameters were measured: (1) infarct size; (2) histological damage; (3) plasma creatine phosphokinase activity; (4) cardiac myeloperoxidase; and (5) cardiac ATP levels. PARS inhibition reduced the infarct size and plasma creatine phosphokinase activity and improved the metabolic status and the histological profile of the reperfused myocardium⁶⁸. The reduction in the activity of cardiac myeloperoxidase after 3-aminobenzamide treatment indicates that PARS inhibitors reduce the

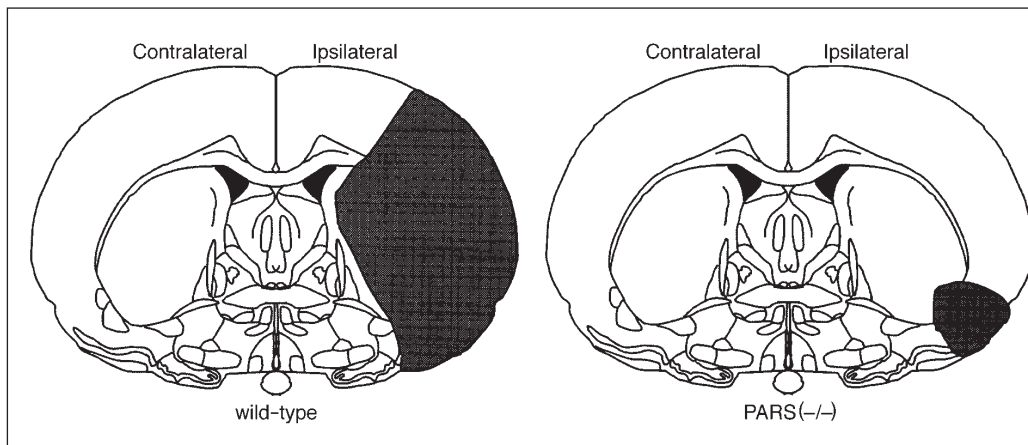
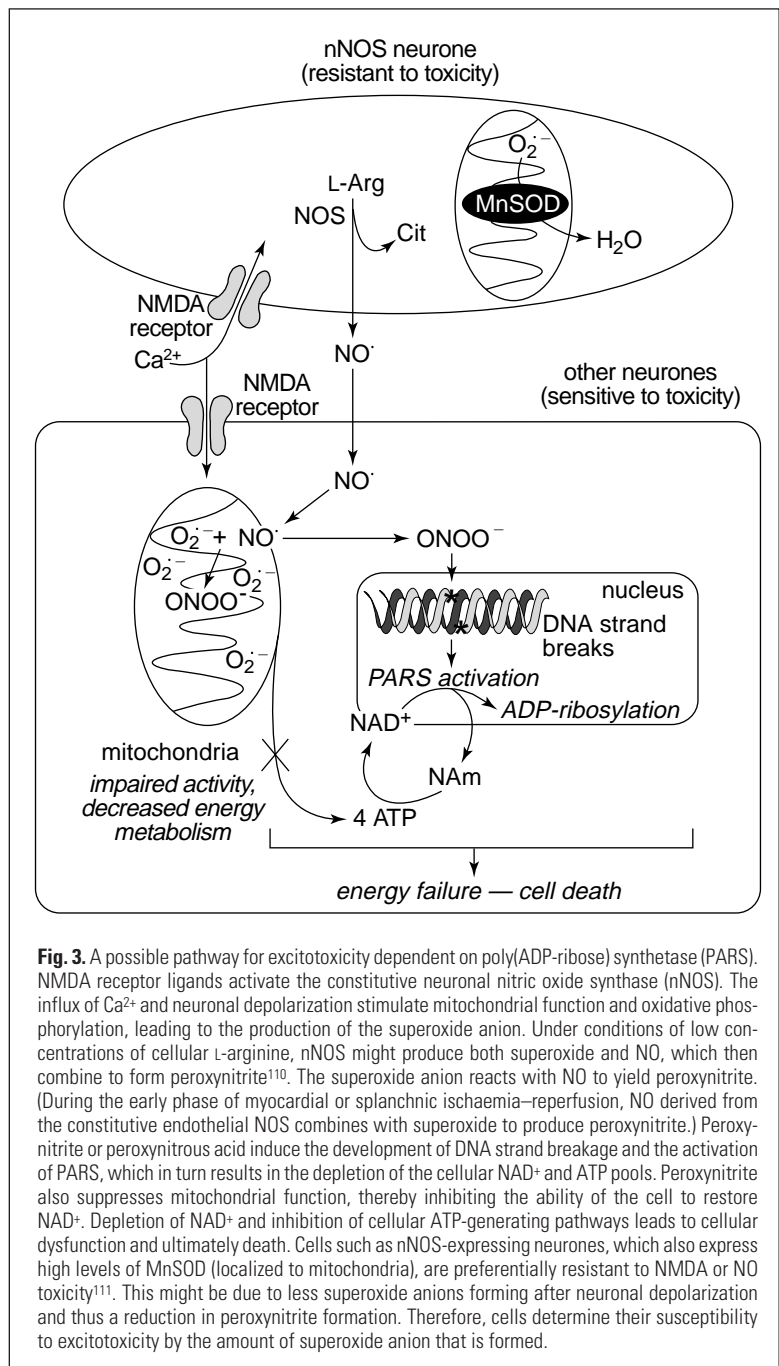
infiltration of neutrophil granulocytes into the reperfused myocardium.

Although 3-aminobenzamide is not a direct scavenger of peroxynitrite and does not inhibit the synthesis or action of its precursors, the PARS inhibitor caused a marked reduction in the amount of reactive peroxynitrite formed in the reperfused myocardium⁶⁸; this observation suggests that the inhibition of PARS, in an indirect way, influences the amounts of reactive peroxynitrite produced. It is most likely that the inhibition of PARS interrupts a self-amplifying, positive feedforward circle, which involves (1) neutrophil recruitment, followed by (2) peroxynitrite formation, followed by (3) endothelial injury and upregulation of adhesion receptors, or both, followed by (4) more neutrophil recruitment. This cycle can be interrupted by PARS inhibitors at the level of endothelial injury³⁷ or at the level of adhesion receptor expression⁹¹. Direct measurements using intravital microscopy of perfused mesenteries have shown that the inhibition of PARS prevents the emigration of adherent neutrophils into the inflamed tissue⁶⁵. This positive feedforward cycle might explain the reduced peroxynitrite generation that was detected in the animal treated with PARS inhibition during myocardial reperfusion⁶⁸ and that also occurs in carrageenan-induced inflammation⁶⁶. It is possible that oxyradical scavengers, which block the increase in myocardial myeloperoxidase activity during reperfusion⁹², also exert their effect by interrupting the same positive feedforward cycle.

The role of PARS activation in ischaemia-reperfusion is not confined to the brain or heart. Recent reports have demonstrated that the inhibition of PARS provides protective effects in the reperfused gut, skeletal muscle and retina. See Table 2 for an overview of the effects of PARS inhibition in a variety of pathophysiological conditions.

PARS and the mode of cell death

In recent years, PARS has been implicated in the process of apoptotic (programmed) cell death. It is suggested that PARS acts as a 'death substrate' for caspases, such as caspase-3 (Ref. 93). Because PARS is assumed to be an enzyme important in DNA repair, the cleavage



Box 1. Additional pharmacological actions of

'Prototypical' poly(ADP-ribose) synthetase inhibitors: benzamide analogues and nicotinamide
Benzamide analogues

During the past two decades, benzamide analogues have been the most frequently used agents to inhibit poly(ADP-ribose) synthetase (PARS) because they display low toxicity *in vivo* and are highly selective for PARS compared with mono(ADP-ribose) transferase. These agents have been used most extensively as commercially available PARS inhibitors to investigate the role of PARS in various experimental systems *in vitro* and *in vivo*.

Benzamide analogues have additional hydroxyl scavenging effects^{1,2} but they do not scavenge NO or peroxynitrite³⁻⁵. Similar to nicotinamide and benzopyrones, benzamides inhibit the expression of inducible nitric oxide synthase (iNOS)^{4,6-9}. Inhibition of the expression of adhesion receptors intercellular adhesion molecule 1 (ICAM-1) and of human leukocyte antigen-DR (HLA-DR) by 3-aminobenzamide has also been reported^{10,11}. Inhibition of gene expression by PARS inhibitors is possibly related to regulation by PARS of the activation of transcription factors c-Fos and c-Jun, nuclear factor κ B, or inhibition of gene expression by other mechanisms^{8,12-14}. Conflicting effects of 3-aminobenzamide on apoptosis have been reported: the drug has been shown to stimulate¹⁵⁻¹⁸ or inhibit¹⁹⁻²¹ apoptosis. The role of PARS in the process of apoptosis might well be dependent on the cell type and on the inducer of the apoptosis.

Contrasting effects of benzamide analogues on the rejoining of breaks in the DNA strand have also been reported: the reports range from stating an inhibition of break rejoining²²⁻²⁴ to lack of effect^{22,25,26}. It has been proposed that benzamides and other PARS inhibitors do not modify the repair of sublethal damage but suppress repair of potentially lethal damage²².

Benzamide analogue PARS inhibitors can also exert significant metabolic effects and thereby inhibit protein, DNA and RNA synthesis (see also main text). 3-Aminobenzamide has also been shown to act as an inhibitor of cytochrome P-450 enzymes; the reduced toxicity to some of the environmental toxins benzamide analogues might be related to a decreased metabolic activation of these substances²⁷.

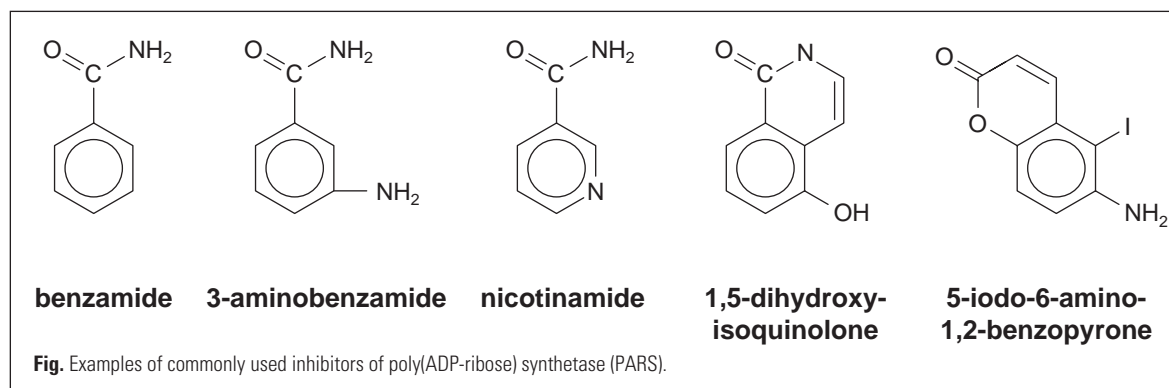
3-Aminobenzamide has been reported to increase the expression of the gene encoding the gluconeogenic enzyme phosphoenolpyruvate carboxykinase in H4IIE hepatoma cells²⁸ and to induce the expression of metallothionein in human tumour cell lines²⁹. Moreover, benzamide alters the hepatic expression of cytochrome P-450, styrene oxide hydrolase, cholesterol oxidase hydrolase, glutathione S-transferase and UDP-glucuronyl transferase³⁰.

Nicotinamide

Nicotinamide, the other most commonly used PARS inhibitor, is less potent than benzamide analogues³¹. Nicotinamide shares most of the characteristics of the benzamide analogues, in that it is an oxyradical scavenger and inhibits iNOS and adhesion receptor expression. Its effects on apoptosis and the rejoining of single-strand breaks in DNA are similar to that of 3-aminobenzamide. Nicotinamide provides the precursor for nicotinamide adenine diphosphate (NAD⁺) biosynthesis and thus enhances the replenishment of the cellular NAD⁺ pools. It has good bioavailability and relatively low toxicity. Nevertheless, it must be noted that caution should be exercised if nicotinamide is given at high doses since it can induce an overproduction of N-methyl-nicotinamide, a depression of the methyl donor status, a decrease in the amount of plasma and liver choline and of liver S-adenosyl-methionine, increases in lipid content and oxidant stress³². Clinical trials with nicotinamide in diabetes mellitus have produced encouraging preliminary results.

Novel, more potent PARS inhibitors: isoquinolines and benzopyrones*Isoquinolines*

1,5-Dihydroxyisoquinoline and its derivatives, such as PD128763, have been identified as potent inhibitors of PARS *in vitro*³³⁻³⁵. This agent can induce almost complete inhibition of cellular PARS activity, which can lead to radiosensitization and an increase in the death of tumour cells in response to irradiation or cytotoxic drugs³³⁻³⁷. In contrast, it appears that incomplete inhibition of PARS does not exert such sensitizing effects because only a minor fraction of PARS activity is sufficient to aid cellular recovery after sublethal



(i.e. the inactivation) of PARS, in turn, would lead to enhanced apoptosis because it would end the 'basal' poly(ADP-ribosylation) of Ca²⁺-Mg²⁺-dependent endo-

nuclease and the ADP-ribosylation of histone H1, which are required for the suppression of apoptosis. In this context, the PARS-related mechanisms of apoptotic cell

poly(ADP-ribose) synthetase inhibitors

oxidant damage³⁶. Much of the reported cellular effects of the hydroxyisoquinolines are related to an enhanced sensitivity of tumour cells to antitumour interventions^{33–37}.

In 1994, Zhang and colleagues found that 1,5-dihydroxyisoquinoline can give protection against NMDA-induced neurotoxicity in cortical cells in culture³. This PARS inhibitor can also provide partial, but significant, protection in a model of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity in mice³⁸. 3,4-Dihydro-5-[4-(1-piperidinyl) butoxy]-1(2H)-isoquinolinone provides protection in a rat model of stroke, with a bell-shaped dose-response curve; thus, at the highest doses tested, the protective effect was diminished compared with that in response to lower doses³⁹. Isoquinoline-derived PARS inhibitors also include H-benz[c]isoquinolin-1-one 6(5H)-phenanthridinone³¹; 6(5H)-phenanthridinone⁴⁰ and the positive inotropic agent vesnarinone⁴¹.

Benzopyrones

Benzopyrone analogues, most notably 6-amino-1,2-benzopyrone and 5-iodo-6-amino-1,2-benzopyrone (INH2BP) display high potencies for PARS, good bioavailability, low toxicity, and were originally developed as antitumour and antiviral agents^{42–44}. Recent studies have also demonstrated their protective effects in various models of cellular oxidant injury and inflammation^{9,45,46}.

Conclusion

Taken together, a variety of agents have been identified for pharmacological inhibition of PARS, and have been used *in vitro* and *in vivo*. The desirable and undesirable side-effects of the 'prototypical' or 'classical' PARS inhibitors (benzamide analogues and nicotinamide) have been widely reported because the compounds have been available for over 20 years. On the contrary, much less information is available on the selectivity and potential side-effects of the more recent, more potent PARS inhibitors (such as the isoquinolines and benzopyrones). At present, only very limited pharmacodynamic and pharmacokinetic data exist for these novel agents. Therefore, appropriate caution should be used in experimental design and in the interpretation of the data obtained using these compounds.

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death are perceived as terminal, delayed effectors⁹⁸. These roles of PARS in promoting apoptosis are probably not universal and might be dependent on cell type

or the stimulus involved: in a number of studies, the inactivation or pharmacological inhibition of PARS was not found to suppress apoptosis^{7,94–97}. In fact, the

activation of caspase-3 has been observed in thymocytes of PARS(-/-) mice exposed to peroxynitrite; the inhibition of caspase-3 suppresses apoptotic DNA fragmentation in these cells, pointing towards the importance of substrates other than PARS in the caspase-3-mediated apoptotic process⁹⁴.

By contrast, in the scheme of PARS activation discussed in this review; that is, as a consequence of DNA injury (Figs 1, 3), the energy depletion induces rapid, necrotic (rather than delayed, apoptotic) cell death, characterized by acute, severe cell injury, changes in membrane integrity, release of lactate dehydrogenase from the cells, and mitochondrial injury^{33,39,40,94,95}. In fact, in thymocytes and other cell types subjected to peroxynitrite or hydrogen peroxide, the inhibition of PARS can divert the mode of cell death from the necrotic towards the apoptotic mode⁹⁴⁻⁹⁶. This phenomenon is consistent with the principle that apoptosis is an energy-requiring process⁹⁸: the maintenance of cellular energy levels (due to the inhibition of PARS) enables the oxidant-treated cells to undergo apoptosis^{94,95}. Based on these studies *in vitro*, we conclude that the mode of action of PARS inhibitors in inflammation and ischaemia-reperfusion is related to protection against free-radical and oxidant-induced cell necrosis (rather than to the inhibition of apoptosis).

The specificity of PARS inhibitors

PARS can be inhibited by a variety of agents, such as benzamide and its analogues (most commonly, 3-aminobenzamide), nicotinamide, benzopyrone derivatives and isoquinolones^{99,100}. The prototypical PARS inhibitors, such as 3-aminobenzamide and nicotinamide, can exhibit a variety of nonspecific pharmacological effects, depending on the agent and the concentration used (see Box 1). For instance, 3-aminobenzamide, at concentrations of 10 mM and higher, has been shown to act as a scavenger of the hydroxyl radical¹⁰¹. Nicotinamide can also act as an oxyradical scavenger. Nicotinamide is the precursor for NAD⁺ biosynthesis and so it might help to replenish the cellular NAD⁺ pools. Additionally, benzamide analogues that are PARS inhibitors can also exert significant metabolic effects and, thereby, inhibit protein synthesis, DNA and RNA synthesis, and the metabolism of glucose, glycine and formate^{24,102-104}. In particular, the inhibition of DNA or protein synthesis is a potential problem for CNS research because inhibition of these activities has been shown to provide neuroprotection in several models, either through sparing the glutathione pool or by inhibiting the expression of 'death' gene products⁸¹. However, the nonspecific effects of benzamides and nicotinamide only become apparent at higher concentrations (1-30 mM)^{24,102-104}. Thus, careful experimental design enables the completion of meaningful experiments with these agents. For example, 3-aminobenzamide at concentrations of 1-5 mM does not appear to inhibit oxidant-induced single-strand breakage of DNA, indicating the lack of significant scavenging

activity²⁴, whereas at higher concentrations (e.g. 10-15 mM and above) the PARS inhibitors can inhibit DNA single-strand breakage due to a direct scavenging effect²⁴. The inclusion of structurally related analogues (e.g. benzoic acid or 3-aminobenzoic acid)¹⁰³ as negative controls is recommended for studies *in vitro* and *in vivo*^{24,37,65,69}.

Only limited published information is available so far regarding the pharmacokinetics of PARS inhibitors, their uptake into various organs, and the length of time they remain inside cells. However, it has been reported that the injection intraperitoneal (i.p.) of 3-aminobenzamide at a concentration of 600 mg kg⁻¹ body mass has a calculated half-life of 90 min in the rat; at 5 min and 4 h after this dose had been administered, the compound was found in the liver at concentrations of 4.5 mM and 1.3 mM, respectively¹⁰⁵. Moreover, the concentration of benzamide has been measured in the mouse brain striatum after systemic administration: following a single i.p. injection of 160 mg kg⁻¹ body mass, benzamide was present in the striatum at a concentration of about 60 ng mg⁻¹ tissue 15-60 min after injection, and was still detectable after 90 min (8.3 ng mg⁻¹ tissue)⁶¹. Thus, the calculated molar concentrations in this tissue ranged from 0.64 to 0.09 mM (Ref. 105). Agents with higher potency and bioavailability for PARS, such as 3,4-dihydro-5-[4-(1-piperidynil) buthoxy]-1(2H)-isoquinolinone or 5-iodo-6-amino-1,2-benzopyrone, might be more selective inhibitors both *in vitro* and *in vivo*^{37,64,77}. However, only a limited amount of information is available at present with respect to the potential additional pharmacological effects of the novel, more potent PARS inhibitors listed above (Box 1). With the emergence of PARS knockout mice^{6,9}, clear-cut comparisons can now be made between wild-type and PARS(-/-) mice in various models of injury in order to test the role of PARS in a given pathophysiological condition. In the first sets of such studies, PARS(-/-) cells were found to be resistant to various types of oxidant injury^{33,37,40,57}, whereas PARS(-/-) animals were found to be resistant against inflammation and organ injury and against ischaemia-reperfusion^{40,63,65,106}.

Concluding remarks and future concepts

It seems surprising that PARS was not deleted during the evolution process because it is an enzyme that can exert marked suicidal effects. Nevertheless, it does not appear to be obligatory for normal development because PARS knockout animals are normal and viable^{6,9}. In PARS(-/-)/129 mice, the lack of PARS does not compromise DNA repair induced by ultraviolet radiation or the alkylating agent MNNG but PARS does play an important role in the maintenance of genomic stability⁶. In a subsequent study, PARS(-/-)/129/C57B6 mice were found to be more sensitive to the methylating agent *N*-methyl-*N*-nitrosourea or to γ -irradiation than their wild-type littermates⁹. The difference between the above two studies might be related to background differences between the two strains 129SV and 129SV/C57B6.

Alternatively, the difference between the two studies might be due to the nature of the DNA-damaging agent, and/or to a difference in the extent of DNA injury. In the study where no difference in sensitivity to γ -irradiation was observed, a dose of 4.5 Gy was used⁷, whereas an enhancement in sensitivity was seen at a dose of 8 Gy (Ref. 9). Based on studies *in vitro*, it appears that PARS inhibition enhances cell death under conditions of severe DNA injury but does not potentiate cell death during less severe irradiation¹⁰⁷. Moreover, it appears that sensitization to the radiation only takes place after complete, but not during partial, inhibition of PARS (Ref. 108). Therefore, it is uncertain whether or not radiation sensitization is a potentially deleterious side-effect of pharmacological PARS inhibition.

Taken together, direct and indirect experimental evidence presented in this review supports the view that DNA strand breakage and PARS activation contribute significantly to the pathophysiology of various forms of inflammation and ischaemia-reperfusion. Therefore, pharmacological inactivation of PARS might be a therapeutically viable strategy to limit cellular injury and improve the outcome of a variety of pathophysiological conditions associated with the overproduction of oxygen- or nitrogen-derived oxidants and free radicals.

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Chemical name

PD128763: 3,4-dihydro-5-methylisoquinolinone

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