Poly(ADP-ribose)polymerase Inhibition – Where Now?

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Abstract: The poly(ADP-ribose)polymerases (PARPs) catalyse the transfer of ADP-ribose units from the substrate NAD+ to acceptor proteins, biosynthesising polyanionic poly(ADP-ribose) polymers. A major isoform, PARP-1, has been the target for design of inhibitors for over twenty-five years. Inhibitors of the activity of PARP-1 have been claimed to have applications in the treatment of many disease states, including cancer, haemorrhagic shock, cardiac infarct, stroke, diabetes, inflammation and retroviral infection, but only recently have PARP-1 inhibitors entered clinical trial.

Most PARP-1 inhibitors mimic the nicotinamide of NAD+ and the structure-activity relationships are understood in terms of the structure of the catalytic site. However, five questions remain if PARP-1 inhibitors are to realise their potential in treating human diseases. Firstly, the consensus pharmacophore is a benzamide with N—H conformationally constrained anti to the carbonyl—aren bond but this is also a "pharmacophore" for insolubility in water; can water-solubility be designed into inhibitors without loss of potency? Secondly, some potential clinical applications require tissue-selective PARP-1 inhibition; is this possible through prodrug approaches? Thirdly, different diseases may require therapeutic PARP-1 inhibition to be either short-term or chronic; are there potential problems associated with chronic inhibition of this DNA-repair process? Fourthly, PARP-1 is one of at least eighteen isoforms; is isoform-selectivity essential, desirable or even possible? Fifthly, PARP activity can be inhibited in cells by inhibition of poly(ADP-ribose)glycohydrolase (PARG); will this be a viable strategy for future drug design? The answers to these questions will determine the future of disease therapy through inhibition of PARP.

Keywords: Poly(ADP-ribose)polymerase, PARP, DNA repair, solubility, prodrug, chronic, isoform, poly(ADP-ribose)glycohydrolase.

INTRODUCTION

It is now over one hundred years since Sir Arthur Harden deduced the existence of nicotinamide adenine dinucleotide (NAD+) during his studies on the metabolism of sugars [1]. In this time, the role of NAD+ in the production and transmission of energy in cells has moved from an innovative discovery to one of the basic concepts in undergraduate and high-school biochemistry courses. In mammalian cells, there are many enzymes that use NAD+ as a substrate or co-substrate. Most are oxido-reductases, including several enzymes involved in generation and transmission of energy and at least one (inosine monophosphate dehydrogenase) [2] which has been a target for cytotoxic drug design for cancer therapy. However, about forty years ago, it was first proposed that NAD+ could act as a substrate and was consumed to make a polyanionic biopolymer, in response to DNA damage. The enzyme responsible, later called ADP-ribose transferase (ADPRT), poly(ADP-ribose)synthetase (PARS) or poly(ADP-ribose)polymerase (PARP) and now known as PARP-1, was initially a biochemical curiosity with the sole role of managing repair of damaged DNA. However, more recent research has shown a variety of roles within the cell, most connected to its catalytic activity [3,4] but some to protein-protein interactions of PARP-1 in recruiting other proteins to the site of damage to the DNA and also in other functions [5-10].

The principal catalytic activity of PARP-1, and possibly other PARPs, upon activation by DNA damage, is to transfer ADP-ribose units from NAD+ to glutamate side-chains of its target proteins, particularly histones [11,12]; additional ADP-ribose units are then added to the 2'-OH of the protein_ADP-ribose to generate poly(ADP-ribose), a biopolymer which can be up to 100 KDa in size and can contain branch points (Scheme (1)) [13-15]. The function of this polyanionic polymer is to unpack the histones from the DNA in the chromatin, allow PARP-1 to leave the site of DNA damage and expose the damaged site to the repair systems [16]. The PARP-1 protein is itself of ca. 116 KDa and has a modular domain structure (Fig. (1)), comprising an N-terminal DNA-binding domain (with two zinc fingers), a central automodification domain and a C-terminal NAD+-binding domain. Although the complete enzyme has not been crystallised, the crystal structure of the latter NAD+-binding catalytic domain of PARP-1 from chicken was reported in 1996 and the data were disclosed in 1997 [17]. This structure has been used extensively for the study of the catalytic mechanism of the enzyme [18], for the design of candidate inhibitors and for ex post facto rationalisation of the binding modes of inhibitors and of structure-activity relationships [19-25].
Scheme 1. Reaction catalysed by PARP enzymes and structure of linear and branched poly(ADP-ribose) (pADPR). When NAD$^+$ binds to the catalytic site, tight hydrogen-bonding with Gly863 and Ser904 may mechanically stretch and weaken the C-N bond, facilitating the leaving of the nicotinamide and generation of the intermediate cyclic oxonium ion. This electrophile is captured by either a carboxylate on the acceptor protein (for the first cycle) or by an OH on the growing pADPR.
The major isoform, PARP-1, has been the target for design of inhibitors for over twenty-five years but why and how should the catalytic activity of PARP or PARP-1 be inhibited? In other words, what are the potential therapeutic benefits of inhibition, what inhibitors are known and what is the pharmacophore required for inhibition? Since PARP-1 (the only isoform known before the late 1990s) is a controlling enzyme in the repair of damage to DNA and radiotherapeutic and many chemotherapeutic approaches to cancer therapy act by inducing DNA damage, it is unsurprising that the first proposed applications of PARP-1 inhibitors were in radiopotentiation and chemopotentiation of cancer treatment. Potentiation of the cell-killing effects of radiation has been demonstrated in vitro and in vivo for a number of inhibitors of varying structure and potency [26-29]. As one might expect, potentiation of therapy by PARP inhibitors has been demonstrated for those agents that act through damaging DNA by diverse mechanisms, such as temozolomide [20-22,30-36], topotecan [20-22,30], irinotecan [36], cisplatin [36], bleomycin [37,38] and MTIC [38] but not by agents, such as the antimetabolites nolatrexed and gemcitabine [38], that act elsewhere in the cell. Indeed, the first report on the involvement of PARP activity and temozolomide was in 1985, when Tisdale noted that this DNA-methylating agent increased the biosynthesis of poly(ADP-ribose) [39]. A clinical trial of a PARP-1 inhibitor was initiated in Newcastle, UK, in mid-2003 to test this chemopotentiation. Interestingly, inhibition of PARP activity has also been reported to be protective against the some side-effects of cancer chemotherapeutic cytotoxins. For example, the cardiotoxicity often seen as a dose-limiting toxicity in treatment with doxorubicin is ameliorated in experimental systems [40,41], as is the nephrotoxicity caused by administration of cis-platin [42].

![Fig. (1). Domain structure of PARP-1.](image)

Sudden reperfusion of ischaemic tissues overloads the hypoxic cells with oxygen (O₂), an oxidising diradical, causing extensive damage to DNA. This, in turn, initiates hyperactivation of PARP, leading to massive depletion of NAD⁺, which is also essential for the energy metabolism of the cell, and hence to death by necrosis [43]. It has been proposed that inhibition of PARP, at least in part, should alleviate this NAD⁺ depletion and protect organs from damage following ischaemia and reperfusion. Protection against organ damage by PARP inhibitors has thus been reported in rodent models of haemorrhagic shock [44-46], stroke [47-49], ischaemic kidney disease [50], ischaemic liver disease [51], myocardial ischaemic events [52-54], ischaemic retinal disorders [55], septic shock [56] and mesenteric ischaemia [57,58]. Interestingly, PARP inhibition has been observed to protect against ischaemic injury during organ transplantation [59,60]. Similar effects have been noted for disruption of the PARP-1 gene in myocardial ischaemia and stroke, indicating that this is the major PARP isoform involved in these processes (vide infra) [61,62]. Beneficial therapeutic activity has also been claimed for PARP inhibition in animal models of NMDA-mediated and MPTP-induced neurotoxicity [63,64] and retinal damage [65], diabetes [6,49,66,67], retroviral infections [68,69], skin senescence and UV-induced skin damage [70,71] and acute and chronic inflammation in various sites in the body (including lung and colon) [72-77]. Interestingly, the potent water-soluble inhibitor 5-AIQ prevents an experimental asthma-like condition in guinea pigs [78] and another PARP inhibitor, PJ34, protects against allergic encephalomyelitis in an animal model [79]. Malfunction of the immune systems often follows profound stress and this immunocompromise is alleviated by inhibition of PARP activity [80]. Pharmacological inhibition of PARP activity and other agents alleviates neurological deficits consequent to spinal cord injury [81-84]. Over-stimulation of PARP activity also contributes to pre-eclampsia in human pregnancy, pointing to another possible application of inhibitors [85]. The potential clinical applications of PARP inhibitors have recently been reviewed thoroughly [86-91], including the patent position [92], and further review is unnecessary here.

It is similarly unnecessary to repeat in detail the excellent review of the design of inhibitors of PARP activity published in 2003 by Southan and Szabó [93] but rather the structure-activity relationships will be outlined briefly. The first selective inhibitor of PARP activity, 3-aminobenzamide (3-AB) was reported some twenty-five years ago by Purnell and Whish [94]; this compound (IC₅₀ 22 μM) was the "gold standard" for PARP inhibition for over a decade. During this time, inhibitory activity was claimed for a wide variety of structures [95], including flavone and 5-iiodouridine but the most active compounds, 4-amino-1,8-naphthalimide, phenanthrindin-6(5H)-one and "1,5-dihydroxyisoquinolino" (actually the 5-hydroxyisoquinolin-1-one tautomer), found in this study contained the constrained arylamide motif which has become the consensus pharmacophore for later drug design. This pharmacophore mimics the nicotinamide moiety of the substrate NAD⁺. Fig. (2) shows the structures of examples of known potent inhibitors, together with the structure of the consensus pharmacophore containing the benzamide with the amide N-H held cis to the amide carbonyl either by incorporation into a covalent five-membered ring (in isoidolones), covalent six-membered rings (isoquinolin-1-ones [26,46,48-52,95,96], 3,4-dihydroxyisoquinolin-1-ones [26,97], quinazolin-4-ones [24,48,97-100], thieneoisouquinolines [48], phthalazin-1-ones [95,97], phthalazine-1,4-diones [95], phenanthrind-6-ones [94,95], naphthalimides [95]) and covalent seven-membered rings (benzazepin-1-ones [48] and the highly potent tricyclic inhibitors recently reported by the Newcastle group [20,21] and the Guilford group [49]). Ingeniously, intramolecular hydrogen bonds have also been used by this
group to maintain the required planar benzamide conformation in their potent benzoxazole-4-carboxamides [97] and benzimidazole-4-carboxamides [22] and by a Japanese group in the new lead inhibitor FR261529, a quinoxaline-5-carboxamide [24,101]. Cantoni et al. had claimed the benzene ring was essential in the pharmacophore and could not be replaced by thiophene [102] but this orthodoxy has been challenged by the observation of potent inhibitory activity for series of aminothiophene-carboxamides, thieno[3,4-c]pyridin-4(5H)-ones, thieno[3,4-d]pyrimidin-4(3H)-ones [103] and thieno[2,3-d]pyrimidin-4(3H)-ones [48]. Interestingly, apparent mimics of a greater part of the NAD⁺ structure, such as tiazofurin (which, after intracellular conversion to tiazofurin adenine dinucleotide, inhibits inosine monophosphate dehydrogenase (IMPDH) very potently [104]), are poor inhibitors of PARP. This is consistent with our molecular modelling studies on docking NAD⁺ into the active site of the chicken PARP-1 [17], which show that optimum binding is only achieved when the C-N bond between the ribose and the nicotinamide is significantly stretched [Threadgill, M. D.; Thompson, A. S. unpublished results]; one may speculate that this stretching (and weakening) of the bond which is broken in the first step of the reaction (Scheme (1)) is, at least in part, responsible for the catalysis by PARP. Costantino et al. have discussed the modelling of PARP-1 inhibitors bound to the enzyme and the consequent structure-activity relationships [19].

There are over one hundred enzymes that use NAD⁺ (or NADH). These can be broadly classified into the oxidoreductases and the ADP-ribosyl transferases; the latter group can be subdivided into the mono-ADP-ribosyl transferases (such as diphtheria toxin [105], cholera toxin [106], pertussis C2 toxin [107] and the eukaryotic mono(ADP-ribosyl)transferase [108] which all transfer a single ADP-
ribose unit onto a nitrogen nucleophile), ADP-ribosyl cyclase (involves in mobilisation of Ca^{2+} from intracellular stores) [109,110] and the isoforms of PARP. Thus, at first sight, selective inhibition of PARP activity might seem a daunting task in drug design, especially in the light of the comment in the first report of the crystal structure of the NAD^{+}-binding catalytic domain of a PARP that "the nicotinamide subsites are very similar [in PARP and the bacterial toxins]" [17]. However, the above observation that tiazofurin and its analogues are highly selective for IMP dehydrogenase vs. PARP, together with the high selectivity of 5-AIQ for PARP vs. diphtheria toxin [96] and the high selectivities seen for several compounds of diverse structures for PARP vs. an avian mono(ADP-ribosyl) transferase (and vice versa) [95], shows that drug design for selectivity of inhibition of PARP is feasible and straightforward. Studies on the conformations of NAD^{+} (and inhibitors such as tiazofurin) required for optimum binding to the range of enzymes indicate that the critical feature is the dihedral angle along the anomeric C-N bond between the ribose C1'-O and the plane of the pyridine (or mimicking heterocycle) [111-113].

Inhibition of PARP activity is a hot topic in current medicinal chemistry and many groups in the academic sector and in the pharmaceutical industry are pursuing the conventional objective of increasing the potency of inhibition of the enzyme activity. In this context, claims for ever-lower IC_{50} values should be compared with caution, since values for the same compound can vary by up to tenfold, depending on the assay system, even when using cell-free preparations of the enzyme. Since uptake into cells varies widely between compounds and many known inhibitors do not pass the cell membrane efficiently, the IC_{50} values for inhibition of PARP activity in whole cells are even more disparate and are usually much higher.

The first clinical trials of PARP inhibitors (the Newcastle tricyclic benzimidazole, Inotek’s INO-1001 and Fujisawa Pharmaceutical’s FR255595) have only recruited their first patients in the last few years, more than thirty years after the discovery of the enzyme. One of these studies has the therapeutic objective of potentiating the anticancer activity of temozolomide but the results are still awaited. However, five questions remain if PARP-1 inhibitors are to realise their potential in treating human diseases. Firstly, the consensus pharmacophore is a benzamide with N—H conformationally constrained anti to the carbonyl—arene bond but this is also a "pharmacophore" for insolubility in water; can water-solubility be designed into inhibitors without loss of potency? Secondly, some potential clinical applications require tissue-selective PARP-1 inhibition; is this possible through prodrug approaches? Thirdly, different diseases may require therapeutic PARP-1 inhibition to be either short-term or chronic; are there potential problems associated with chronic inhibition of this DNA-repair process? Fourthly, PARP-1 is one of at least eighteen isoforms; is isoform-selectivity essential, desirable or even possible? Fifthly, PARP activity can be inhibited in cells by inhibition of poly(ADP-ribose)glycohydrolase (PARG); will this be a viable strategy for future drug design? The answers to these questions will determine the future of disease therapy through inhibition of PARP.

**THE NEED FOR WATER-SOLUBLE PARP-1 INHIBITORS**

The intensive work over the last few years has seen the emergence of several classes of highly potent PARP-1 inhibitors. However, in the passionate quest for greater potency, relatively much less effort has been expended on other equally important areas of drug development, such as tissue selectivity, bioavailability and water-solubility. Most of the PARP-1 inhibitors reported to date are structurally based on the pharmacophoric benzamide planar ring system, which, ironically, is also a "pharmacophore" for water-insolubility [95]. As a result, many of these otherwise highly promising inhibitors, such as DPQ (IC_{50} 0.04 μM), PND (IC_{50} 0.30 μM) and GPI-6150 (IC_{50} 0.06 μM) suffer from poor water-solubility and this gives rise to various inherent problems (Fig. 3).

![Fig. 3. Structures of potent PARP inhibitors DPQ, PND and GPI-6150, which suffer from poor pharmaceutical properties.](image-url)
water-soluble is crucial. The question, then, is can water-solubility be designed into such inhibitors without a loss of potency, given the hydrophobic nature of the pharmacophore and the tendency of compounds bearing this secondary amide to form hydrogen-bonded dimers in the crystalline state, leading to high-melting crystalline solids? [18]. This probably contributes to the increased potency for the larger, planar fused ring molecule but at the expense of its water-solubility. However, opportunity for improvement of their pharmaceutical characteristic with possible further enhancement of their potency arises with recent X-ray crystallographic data demonstrating putative water-mediated hydrogen-bond interactions between Glu988 of the active site and the amino group of 4-amino-1,8-naphthalimide (IC50 0.18 µM) [19]. This amino group was also found to correspond, approximately, to the 5-position of isoquinolin-1(2H)-one, a related class of inhibitors. In response to this new possibility, our attention was being directed towards 5-AIQ (Fig. (4)).

5-AIQ was first reported by Suto et al. in 1991 to inhibit PARP activity in a cell-free preparation from calf thymus (IC50 240 nM) [26]. As 5-AIQ was not commercially available then, we optimised its synthetic route (previously described by Wenkert et al. [125]) and then converted it to its highly water-soluble hydrochloride salt, 5-AIQ.HCl [46]. This enabled us, in collaboration with several other research groups, to investigate for the first time, its pharmacological effects in a wide range of diseases in vivo, including animal models of myocardial infarction [52], ischaemia-reperfusion of the liver [51] and kidney [50] and acute lung inflammation [74]. In all of these studies, 5-AIQ.HCl was found to exhibit tremendous therapeutic benefits.

Fig. (4). Structures of PARP inhibitors with good water-solubility as their salts; the primary amine may also participate in hydrogen-bonding to the enzyme active site.

Current enzyme-inhibitor interaction studies support the notion that the presence of a planar electron-rich aromatic ring is needed to enhance the ability of the carbonyl group to participate in hydrogen-bonding within the active site. In addition, it also participates in π-π interactions with the phenyl rings of two parallel tyrosine residues (Tyr907 and 896) within the active site, forming a "π-electron sandwich"

Fig. (5). Views from a modelling study of 5-AIQ docked into the nicotinamide-binding site of PARP-1 from chicken. A: View showing hydrogen bonds from the constrained secondary amide to Gly863 and Ser904. B: View showing the π-electron sandwich with 5-AIQ between Tyr 896 and Tyr 907. C: View showing proximity of 5-NH2 group of 5-AIQ to the active site Glu988 carboxylate, allowing possible water-bridged hydrogen bond.
Particularly noteworthy are its protective effects on ischaemia-reperfusion injury caused by severe haemorrhage and resuscitation in anaesthetised rats where it demonstrated exceptional potency in abolishing multiple organ injury and dysfunction [46]. Compared to the benchmark inhibitor 3-AB, which required a dose of 10 mg Kg\(^{-1}\) i.v., only a remarkably low i.v. dose of 30 \(\mu\)g Kg\(^{-1}\) is required to confer similar protection. The concentration at which 5-AIQ.HCl provides protection against oxidative injury (0.1 - 0.3 \(\mu\)M) \textit{in vitro} does not appear to be consistent with its unusually high \textit{in vivo} efficacy. It appears that 5-AIQ.HCl gains much of its advantage over other PARP-1 inhibitors through its excellent water-solubility, which conferred it with favourable pharmacokinetics, such as good absorption and biodistribution. It is also likely that the 5-amino group in 5-AIQ may display similar water-mediated hydrogen-bond interactions with the active site (Fig. (5)), thereby contributing to its exceptional PARP-1 inhibitory potency \textit{in vivo}.

The success of 5-AIQ has paralleled efforts to improve the biopharmaceutical properties of other classes of PARP-1 inhibitors. Several large, polycyclic PARP-1 inhibitors have now undergone structural elaboration to enhance their water-solubility without adversely affecting their potency with varying successes (Fig. (6)) [22,47,60,126]. These inhibitors either have an amino-containing side chain, such as phenylamine, alkylamine and cyclic amines (piperazine, morpholine), a hydroxyl group or an acid-containing side chain, all of which could subsequently be derivatised to form water-soluble salts. For example, both NU1085 (K\(_i\) 6 nM) and PJ34 (EC\(_{50}\) 40 nM) have retained potency and relatively good solubility in water.

Incidentally, for PARP-1 inhibitors to be useful for treatment of neurodegenerative disorders such as Parkinson’s disease, they have to be able to cross the highly lipophilic blood brain barrier. Hence it is important to build both water-solubility and brain-penetration, which are two seemingly contradictory properties, into the same molecule. This is wonderfully illustrated in the recent ingenious discovery of an orally active and brain-penetrable quinazolinone PARP-1 inhibitor by Hattori \textit{et al.} [127]. They discovered that while the quinazoline-2,4-dione 2 (Fig. (7)) exhibits great potency against PARP-1 with IC\(_{50}\) 60 nM, it showed poor brain penetration (0.6 \(\mu\)g g\(^{-1}\)) after intraperitoneal administration in mice. Quinazolinone 3, on the other hand, has an appropriately 10-fold higher brain concentration (5.5 \(\mu\)g g\(^{-1}\)), but suffered from poor potency (IC\(_{50}\) 1200 nM). In an attempt to combine the desired biopharmaceutical properties of quinazolinone 3 with the potency of 2, they designed quinazolinone 4, which is a structural hybrid of 2 and 3, and found that its hydrochloride salt shows strong potency (IC\(_{50}\) 1.1 nM), good water-solubility (5.6 mg mL\(^{-1}\)), good oral bioavailability (70\%), and a high brain/plasma concentration ratio of approximately five. It was also found to protect significantly in a murine model of MPTP-induced Parkinson’s disease.

Hence, despite apparent difficulties, it is possible to build water-solubility into the pharmacophore and, with appropriate structural manipulation, one is also able to incorporate other desirable biopharmaceutical properties into the molecules to enable them to reach specific target tissues. While achieving a greater potency is a worthwhile effort, it has to be carefully considered in the light of tissue penetration and specific pharmacokinetic and pharmacodynamic requirements.
THE NEED FOR TISSUE-SELECTIVE PARP-1 INHIBITION

Ideally, besides being able to discriminate between PARP-1 and other poly-/mono-(ADP-ribosyl)ating enzymes (i.e. enzyme selectivity), PARP-1 inhibitors should also demonstrate tissue selectivity, since the intensity of their pharmacological response and their actual therapeutic efficacy depend largely on their concentration at the desired site of action. Accordingly, a PARP-1 inhibitor that is able to identify and accumulate exclusively within the target tissues will be required at a much lower dose and, consequently, can to reduce the risk of adverse effects and toxicity to other tissues. This is important, since many PARP-1 inhibitors are planar cyclic systems. Such molecules may be prone to DNA intercalation and may, in theory, be associated directly with genotoxic effects, in addition to any risks inherent in chronic inhibition of surveillance and repair of the genome.

Tissue-selectivity is also particularly important for some potential clinical applications of PARP-1 inhibitors, such as sensitising cancer cells towards chemotherapy and radiotherapy. The rationale for their use comes from the observation that PARP-1 is important for DNA base-excision repair and its inhibition causes a significant delay in the DNA repair processes. Accordingly, PARP-1 inhibitors, by preventing tumour cells from repairing DNA, would potentiate the cytotoxic effects of both DNA-acting cytotoxins and ionising radiation used in the treatment of cancer. Indeed, several lines of evidence now indicate that tumour cells can be sensitised by PARP-1 inhibitors to N-methyl-N-nitrosourea, bleomycin, camptothecin and ionising radiation-induced cytotoxicity [28,30]. However, one major concern for this approach is the lack of selectivity of PARP-1 inhibitors (as well as the anticancer agents) for cancer cells. As a result, the DNA repair process for rapidly dividing normal cells that have been exposed to the cytotoxic agents will also be impaired along with those of the tumour cells, thus limiting their effectiveness and possibly giving rise to more severe adverse effects.

To address this problem, various strategies have been devised to deliver PARP-1 inhibitors selectively to the desired site of action. These include formulating PARP-1 inhibitors for localised administration to reduce their systemic absorption and hence, systemic side-effects. For instance, Farkas et al. have prepared a cream formulation of BGP-15M for dermal application to study its protective effects on UV-induced photo damage on the skin and skin cancer [71]. They then determined the concentration of BGP-15M in the skin, serum and muscle on the test animals and they found that the drug was predominantly retained in the skin, with negligible transdermal absorption. They also found that UV light exposure promoted the accumulation of the inhibitor in the skin. It is plausible that this strategy could be applied to specifically target other tissues or organs, for example metered-dose inhaler (MDI) and eye ointment to target ischaemia-reperfusion injuries and inflammatory conditions of the lungs and eyes, respectively.

Another approach includes the use of a prodrug which, in itself, is biologically inactive but could be selectively unmasked or activated by the very tissues on which it is intended to act. Such systems usually employ biochemical or physiological differences between the normal and the pathogenic tissues and examples are the bioreductive prodrugs of PARP-1 inhibitors recently developed in our laboratory. The inspiration for the design came from the observation that many disease states where PARP-1 inhibition is therapeutically beneficial, such as cancers, inflammatory disorders and ischaemia-reperfusion injuries are marked by acute or chronic tissue hypoxia [128-131]. Such a physiological difference in the concentration of oxygen between normal and hypoxic tissues was then exploited through the design of biologically inactive prodrug systems which, upon selective bioreduction in hypoxic tissue, would release PARP-1 inhibitors only in that tissue. Denny has described the modular nature of the design of prodrugs as comprising a Trigger (a substrate for the endogenous or exogenous activating enzyme or physicochemical activating event) and an Effector (the active drug to be released), joined by a Linker which releases the Effector in response to the Trigger (Fig. (8)) [132,133].

The Bath group have studied four different redox-sensitive Triggers for release of isoquinolin-1-one PARP inhibitors. These fall into two groups: the nitroheterocyclimethyl- and 4,7-dioxoindole-3-methyl-types, derived from the nitroheterocycle and mitomycin classes of bioreductively activated cytotoxins, respectively. In each case, the release of the isoquinolin-1-one Effector was initiated by chemical reduction of the Trigger, designed to mimic bioreduction in hypoxic tissues. Selective reduction of the nitro group of the N-(5-nitrofururan-2-ylmethyl)isoquinolin-1-one 5 with the sodium borohydride / palladium / aqueous propan-2-ol system caused release of isoquinolin-1-one 7 within 5 minutes [134]. As shown in Scheme (2), reduction of the nitro gives the amine 6 (R = H); this electron-rich group then feeds its lone pair into the ring, expelling the isoquinolin-1-one leaving group. As designed, the pharmacophore in 5 is truly masked and its PARP inhibitory potency is much weaker. In hypoxic tissue, bioreduction (mediated by cytochrome P450 reductase) either by six electrons to the amine 6 (R = H) or by four electrons to the hydroxylamine 6 (R = OH) should trigger the release. The redox potential of nitrofurans is sufficiently high to raise questions about the possibility of reductively-triggered release in normal oxic tissue [135]. 2-Nitroimidazoles, including the radiosensitising drugs misonidazole and etanidazole, have $E^\circ_{1/2}$ ca. -389 mV, which is more appropriate for selectivity of bioreduction in hypoxic tissue [136]. 2-Bromoisoquinolin-1-one was chosen as the Effector for preliminary studies with this Trigger. A challenging synthesis gave 5-bromo-N-(2-nitroimidazolylmethyl)isoquinolin-1-one 8 [137]. In this case, the borohydride / palladium system was insufficiently selective, in that it triggered release but also caused hydrogenolysis of the C-Br bond; a zinc / ammonium chloride reductant system was more selective in reducing the nitro group only and triggering release of the potent PARP inhibitor 5-bromoisoquinolin-1-one 9. Alkylations of isoquinolin-1-
ones with a 4,7-dioxindole-3-methanol derivative under Mitsunobu conditions gave the N-linked 10 and O-linked prodrugs 13 [138]. Interestingly, reductively triggered release of the corresponding isoquinolin-1-ones 12 only occurred from the O-linked isomers 13 and not from 10 [139], presumably owing to different lifetimes of the intermediate hydroquinones 11 and 14. Similar reduction of the O-linked prodrugs 1-(5-nitrothiophen-2-ylmethoxy)isoquinolines also effected efficient release of the corresponding PARP inhibitors [140]. It will be most exciting to investigate the in vivo release profile of these prodrugs in animal models, once water-soluble analogues have been developed (see above); using these systems, the water-solubilising moieties could be attached to the masking group and not to the pharmacaphore. Incidentally, these highly effective and versatile Triggers have potential for being adapted to other structurally related PARP-1 inhibitors, such as phenanthridinones and quinazolinones.

Another successful prodrug of a PARP-1 inhibitor, which takes advantage of the presence of nitro-reductase activity in malignant cells, is 4-iodo-3-nitrobenzamide (INO2BA). This inactive nitro-precursor was reported by Bauer et al. to be selectively retained and reduced to the highly reactive and tumouricidal nitroso-compound, INOBA, within the E-ras 20 tumour cell line [141]. Such selectivity for cancer cells is evidenced by the complete lack of reduction of the nitro to nitroso group in non-malignant CV-1 cells. They proposed that the nitroso-drug binds to the unconventional second binding site of PARP-1 (which is distinct from the usual NAD⁺ binding domain) and oxidises the asymmetric zinc fingers of PARP-1, causing zinc ejection and PARP-1 inhibition. The action of this inhibitor could be further augmented with the simultaneous administration of buthionine sulfoximine (a known inhibitor of GSH) which serves to remove the nitroso-scavenging effect of GSH.

In view of the preliminary successes achieved in vitro with these prodrug systems, it is conceivable that the use of such tissue-selective prodrugs would dramatically reduce their potential toxicity on normal tissues and increase their

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**Scheme 2.** Demonstration of reductively triggered release of isoquinolin-1-one PARP inhibitors from N-nitrofuranyl methyl 5, N-nitroimidazoylethyl 8 and O-(4,7-dioxindol-3-methyl) 13 prodrug systems. Release from reduced N-(4,7-dioxindol-3-methyl) isomers 10 failed. R = H or OH; R^5 = e.g. I, H, Br, NHBoc.
therapeutic efficacies. It may also make possible the long-term use of PARP-1 inhibitors in certain chronic diseases. This is discussed in greater depth in the next section.

DOES CHRONIC INHIBITION OF PARP CARRY RISKS?

It is unlikely that chronic inhibition of PARP activity in the treatment of cancer, whether to potentiate cell killing by radiation or by conventional DNA-damaging chemotherapy, will be necessary. Moreover, given the life-threatening nature of the disease, the modest outcomes of current therapy for some cancers and, indeed, the considerable risks of toxicity and (in the long term) carcinogenicity from some cytotoxic drug regimens, it could be argued that the risks deriving from therapeutic inhibition of PARP activity would not add significantly to the overall risk to the patient. Of course, no clinical trial has yet been completed, to the risks of short-term treatment with a PARP inhibitor in man are currently unknown; the results of the ongoing clinical trials by the Newcastle group and others are eagerly awaited in this context. Similarly, acute treatment of ischaemia-reperfusion events, such as haemorrhagic shock, septic shock, stroke and myocardial infarction, with PARP inhibitors is probably unlikely to result in adverse effects deriving from inhibition of the enzyme.

In contrast, treatment of chronic diseases, particularly those involving inflammation, may involve pharmacological inhibition of PARP over a considerable period of time, possibly years or for life. Of course, no data are yet available from chronic clinical trials but one may use such animal and in vitro data as are available to understand and predict possible risks; understanding of these potential risks may guide the vigilance necessary in any future clinical trial of chronic administration of a PARP inhibitor.

What are these potential risks and what is the evidence which enables consideration of them? Firstly, since PARP-1 (and probably some other PARPs) are involved in repair of damaged sites in DNA, it is clear that inhibition of PARP activity would result in persistence of this damage. Thus PARP inhibition may be associated with higher levels of mutation and other instability of the genome [142] indeed, PARP has been described as the "guardian angel protecting the genome" [3]. There are very few reports of pharmacological inhibition of PARP with a small-molecule agent causing increased mutagenesis or chromosomal instability; in a comparison of the yield of sister chromatid exchanges and micronuclei (as markers of mutagenesis), it appears that the modest PARP inhibitor 3-aminobenzamide does increase mitomycin C-induced damage (in normal lymphocytes but not in cells from Downs Syndrome patients) [143]. However, studies using trans-dominant inhibition of PARP are more clear; this mode of inhibition does potentiate mutagenesis in several cell types by several mutagens [144,145]. PARP-1 knockout mice also show increased mutation rates after challenge with a variety of alkylating agents [146]. Correspondingly, an increase in the amount (and, presumably, the activity) of PARP through over-expression can protect against mutagenesis [147,148]. Inorganic arsenic compounds are known to potentiate mutagenesis caused by UV light, inter alia. Interestingly, it has been proposed that the mechanism of this potentiation involves inhibition of PARP activity though binding of the arsenic to cysteine sulfur in the zinc fingers, preventing binding of PARP to DNA, preventing recognition of damaged sites and preventing the consequent activation of PARP [149]. It is unclear whether or not this target on the enzyme can be exploited in rational drug design, rather than the more usual NAD+-binding site.

Does this increased sensitivity to mutagenesis of cells with lowered PARP activity translate into increased sensitivity to carcinogenesis? The evidence here is mixed, although there is strong tendency towards connecting lowered PARP activity with increased risk of developing cancer (first noted by Bürkle in 1992 [150] and reviewed recently by Bernstein et al. [151]). PARP-1 knockout mice have a strongly increased risk of developing cancers when challenged with the chemical carcinogens nitrosamines and azoxymethane [152,153]. These observations translate into the clinic, in that one study has shown that patients predisposed to colon cancer (familial adenomatous polyposis; FAP) have cells in which PARP activity is not stimulated by DNA damage caused by 60Co γ-radiation [154]. These patients, with fixed low PARP activity, may be unable to respond appropriately to DNA damage caused by environmental carcinogens and thus be more susceptible to developing cancer. PARP activity also protects against carcinogenesis caused by mechanical agents, such as asbestos, and inhibition of PARP (in this study, by 3-methoxybenzamide) may potentiate the DNA-damage caused by asbestos in pleural mesothelial cells [155]. All these individual pieces of evidence point to PARP activity protecting against the effects of carcinogens and suggest that chronic pharmacological PARP inhibition may carry increased risk of carcinogenesis caused by environmental carcinogens. However, Martin-Oliva et al. have shown that abolition of PARP impairs the promotion of skin cancer through inhibition of the activation of NF-κB and suggest that pharmacological PARP inhibition may be protective against skin carcinogenesis [156].

PARP inhibition has been clearly shown to be anti-retroviral [69,157], probably through inhibition of integration [158,159] (although this has very recently been challenged [160]), but what effect does it have on infections with DNA viruses? Surprisingly, the number of integrations of viral DNA from DNA viruses is increased by pharmacological inhibition of PARP activity, particularly under conditions of oxidative stress [161,162]. Since infection of the liver with DNA viruses is linked to the development of hepatocellular carcinoma [162,163], this may indicate another possible risk derived from chronic therapeutic inhibition of PARP.

The changes in activity of PARP with age were reviewed in 1998 by Bürkle [164]; generally, the activity increases with age in mammals [165,166]. Significantly increased activity has been noted for persons over 100 years of age [167] and, more recently, in organs donated for transplantation from older donors [168]. However, in rats, inhibition of PARP activity with PJ34 has been shown to improve vascular endothelial dysfunction associated with aging [169]. Thus one may speculate that the increased PARP activity in older persons may be either a blessing or a curse.
To summarise, risks of increased development of tumours from exposure to environmental chemical and physical carcinogen, as well as virally-induced tumours may, in principle, arise from long-term therapeutic inhibition of PARP activity. The involvement of PARP-1 activity in learning and memory in primitive organisms [170] may prompt study of the effects of PARP inhibition in these areas in mammals. The significance and size of these risks are currently unquantifiable, in the absence of data from chronic PARP inhibition in humans or other long-lived species (other than the above evidence from the FAP patients). PARP inhibitors, such as INO-1001, have been administered chronically to rodents [171] but there are no reports of adverse effects. Perhaps the "chronic" timescale in mice was insufficiently long to allow development of adverse effects and the true test will come in clinical trials.

IS SELECTIVE INHIBITION OF THE PARP ISOFORMS NECESSARY, DESIRABLE OR EVEN POSSIBLE?

For over thirty years from the initial discovery of PARP activity in the late 1960s and the isolation and characterisation of the PARP enzyme in the 1970s, there appeared to be only one enzyme (now known as PARP-1) responsible for biosynthesis of poly(ADP-ribose) in response to DNA damage. Thus drug design was targeted at inhibiting this PARP and the selectivity required was that agents should inhibit this one PARP without inhibiting the other NAD+ -binding enzymes (see above). One of the first indications that the reality was not quite that simple came from the development of PARP-1 (-/-) knockout mice [172]. These mice are viable and develop normally, despite the previous indications that PARP activity is involved in development, but display an increased risk of skin lesions in old age. The role of PARP in apoptotic cell death was also challenged by Leist et al. in 1997, who noted that wild-type mouse cells and corresponding cells from PARP-1 (-/-) mice underwent apoptosis equally, when triggered by a variety of agents [173]. Finally, Shieh et al. discovered, in 1998, that cells from PARP-1 (-/-) mice do biosynthesise poly(ADP-ribose) which is similar to that biosynthesised by PARP-1 and is similarly degraded by poly(ADP-ribose) glycohydrolase (PARG). This PARP-like activity was triggered by DNA damage. These workers presciently concluded that "these results suggest the presence of a previously unreported activity capable of synthesizing ADP-ribose polymers in PARP-/- cells" [174]. Shall and de Murcia also posed the question "Poly(ADP-ribose) polymerase-1: what have we learned from the deficient mouse model?" in a review in 2000 [175], indicating that PARP-1 is not alone and is by no means the only important PARP in mammalian cells.

The issue of PARP activity independent of PARP-1 was resolved in 1998 by the characterisation of PARP-like activity in a protein, tankyrase, located in complexes attached to human telomeres [176], in 1999 with the identification of a second PARP, PARP-2, by Amé et al. [177] and in 1999 by the observation of PARP-like catalytic activity in the 193 KDa vault protein [178]. The existence of a PARP-3 was also proposed during 1999 [179]. Searches of protein databases looking for homology with the PARP-1 catalytic (NAD+-)-binding domain have revealed eighteen putative PARPs to date [89,180], of which several have been fully characterised as proteins with poly(ADP-ribose)ylation activity and for which roles within the cell have either been fully identified or proposed. Given the number of PARP isoforms which have now been characterised, three questions arise: (i) Do any of the isoforms (except PARP-1) present themselves as new targets for drug design? (ii) Is it necessary, from the drug-safety point of view, to design and develop drugs which are selective for inhibition of one or more of the isoforms? (iii) Given the similarity of the NAD+-binding site sequences of the PARP isoforms, will it be possible to design truly selective inhibitors?

Amongst the PARPs, PARP-2 has probably the closest resemblance to the archetype PARP-1 in its structure and function. The PARP-2 gene encodes a 62 KD protein, which lacks the automodification domain of PARP-1 and has a highly shortened and modified DNA-binding domain. Like PARP-1, it is located primarily within the nucleus [181,182] and can poly(ADP-ribose)late histones in response to DNA damage, although its primary target in this activity is histone H2B [89], rather than histone H1 which is the principal hetero-target of PARP-1 [12]. Interestingly, PARP-2 can act enzymically, like PARP-1, as a homodimer or as a heterodimer with PARP-1 [183] and it is not yet clear whether or not this heterodimerisation is its major function. Both isoforms have been implicated in the cellular response to DNA damage in mammalian cells [184-186], in NO toxicity [186], in chromosomal stability [187]. Thus most of the available evidence ascribes roles for PARP-2 which are similar to those of PARP-1 and one could thus argue that the development of specific PARP-1-selective or PARP-2-selective inhibitors was not justified, as what is required for the various therapeutic applications would be inhibition of both isoforms. PARP-2 can compensate in many ways for deficiency of PARP-1 [188]. PARP-1 knockout mice are viable [172], as are PARP-2 knockout mice, but the double knockout genotype PARP-1-/-/PARP-2-/- is lethal. Similarly, PARP-2 is cleaved during apoptosis, although by caspase 8, rather than caspase 3 which is primarily responsible for cleaving and inactivating PARP-1 [189].

However, additional roles for PARP-2 are hinted at by the location of some PARP-2 protein at centromeres [190] and by its apparent negative regulation of TRF2 and consequent involvement in maintenance of telomere length [172]. The latter observation might be thought to provide a potential therapeutic application for a selective PARP-2 inhibitor but the biochemical, signalling and regulatory systems are complicated by the existence of two further PARPs (the tankyrases; see below) which have their principal roles as elements of the telomere regulatory apparatus.

What then would be the benefits of inhibition of PARP-2? In the absence of highly selective small molecule inhibitory agents, one has to turn to antisense oligonucleotide approaches to inhibit its expression selectively to start to address this question. The few pieces of evidence so far indicate that this mode of inhibition does have beneficial effects on colonic function and inflammation in mouse models of colitis, even in the absence of PARP-1...
PARP-2 is a valuable therapeutic strategy for any disease. It is far from clear whether or not selective inhibition of PARP-2 will give valuable insight into the function of PARP-2 but, at present, PARP-2 should be feasible, selective inhibitors will give valuable insight into the function of PARP-2 and of potential therapeutic opportunities which may be presented by selective inhibition of this isoform will require development of much more selective inhibitors than are currently available. Perkins et al. [194] have developed a yeast-based screen for identifying isoform-selective inhibitors of PARP-1 and of PARP-2; this assay is based on heterologous expression of either of the isoforms in Saccharomyces cerevisiae and the convenient event-point is growth inhibition. Using the assay, compounds with up to eight-fold selectivity for PARP-1 (ICX56290675) and three-fold selectivity for PARP-2 (ICX56258231, possibly a hydrolytically activated prodrug for the corresponding phthalazinone 15 for which no comparative data are available; Scheme (3)) were identified; these selectivities are relatively modest. The EC50 values are in the 5-60 µM range in these cells but EC50 / IC50 values in cells are frequently in the µM range for PARP-1 inhibitors which can have PARP-1 inhibitory IC50 values 10-100 times lower in assays in cell-free systems. Ishwita et al. also noted some selectivity for inhibition of PARP-2 (IC50 7 nM in a cell-free assay) over PARP-1 (33 nM) by their new agent FR261529 [101]. In the last few months, this group have also reported similar compounds which have up to twelve-fold selectivity for PARP-2 inhibition by the quinoxalinicarboxamide 16 (Scheme (3)) and up to forty-fold selectivity for PARP-1 inhibition by the quinazolinone 2 (Fig. (7)) [24]. These selectivities are a good starting-point but may not have been achieved by rational design of the molecules but by screening of existing agents. Now that the X-ray crystal structure of the catalytic NAD+-binding domain of murine PARP-2 is available [195], medicinal chemists are presented with an excellent opportunity of structure-based drug design of much more selective inhibitors; although the nicotinamide-binding slots are similar, there exist sufficient differences to make feasible the design of PARP-2-selective inhibitors. Selectivities of at least 100-fold for PARP-4 or for PARP-2 should be the target for the development of small-molecule pharmacological tools which will help to dissect differences in the roles of the two isoforms in the cell. Thus selective inhibition of PARP-2 should be feasible, selective inhibitors will give valuable insight into the function of PARP-2 but, at present, it is far from clear whether or not selective inhibition of PARP-2 is a valuable therapeutic strategy for any disease.

PARP-3 was also first characterised from the cDNA that initially revealed PARP-2 [178]. Much less is known about the functions of this isoform, which is severely truncated from the N-terminal with respect to PARP-1 and is of only 67 KDa in size, corresponding to 540 amino-acids. The NAD+ -binding domain is retained and has catalytic poly(ADP-ribosyl)ating activity, at least in autodigestion, but it appears not to have any role in protection against DNA damage and is not activated by single-strand breaks. The short N-terminal domain of 54 amino-acids causes it to localise at the centrosome. Here it has been shown to be able to interact with PARP-1. Overexpression of PARP-3 interferes with the G1→S step in the cell cycle. It has been proposed [196] that one of the functions of PARP-3 may be to attract and bind PARP-1 to the centrosome to link the surveillance and repair processes mediated by PARP-1 to the mitotic fidelity checkpoint. The distribution of PARP-3 between tissues is not uniform, with high levels in muscle, lung, liver, kidney and ovary and almost no protein in brain and testis [197]. No crystal structure is currently available for PARP-3 and no effort has yet been reported directed at synthesis and identification of PARP-3-selective inhibitors, perhaps in view of the paucity of understanding of its roles and of potential therapeutic applications.

Vaults are large (65 × 35 nm) ribonucleoprotein particles found mainly in the cell cytoplasm [198,199]. The name arises from the shape of these particles, which is of a hollow barrel with multiple arches around the sides, reminiscent of the vaulting in a cathedral. The structure of the vaults has 48-fold rotational symmetry, as determined by cryo-electron-microscopy [200]. There are three vault proteins, vPARP (PARP-4), major vault protein (MVP) and TEP1, and non-coding vRNA within the structure [178,199-201]. Although vPARP has a structural and scaffolding role in vaults [201], early studies show that it can poly(ADP-ribosyl)ate itself and MVP [178], although this is challenged by Rossi et al. [202]. The exact role of vPARP is, however, somewhat complicated by its additional demonstrated association with telomerase [203] but the physiological relevance of this association is not clear.

From the therapeutic point of view, the importance of vaults (and thus vPARP) is their involvement in some types of multi-drug resistance (MDR) to anticancer drugs [204-
In the lung, the lung resistance-related protein has been shown to be identical to MVP and there are clinical data to connect the level of expression of MVP to response to chemotherapy [206]. A more detailed study by Siva et al. showed that up-regulation of vaults, although observed and apparently necessary for MDR, is not sufficient on its own to confer this phenotype, implying that there is a requirement of an additional factor or factors for vault-mediated MDR [207]. One might therefore speculate that inhibition of the up-regulation of vaults or disruption of their assembly, possibly through interfering with the activity of vPARP, may be a useful therapeutic strategy to combat MDR in cancer chemotherapy. However, a useful reminder that vaults are by no means the only mediators of MDR was provided recently by van Zon et al., who confirmed that the increased efflux rate of daunorubicin from cells with an MDR phenotype is independent of the expression of vault proteins [208]. No small-molecule inhibitors have yet been reported for the (controversial) poly(ADP-ribosyl)ating activity of vPARP and no structure is yet available for the protein to facilitate the design of selective inhibitors [200]. A decision on whether or not it will be a worthwhile goal to develop such inhibitors will have to await deeper and clearer understanding of the molecular function(s) of vPARP within vaults and of the detailed role(s) of vaults themselves in resistance to drugs and drug efflux from cells.

The earliest "alternative" PARP to be discovered was tankyrase-1 (also known as PARP-5), through observation of PARP-like activity at human telomeres [275], although the same group later noted the presence of some tankyrase-1 at centrosomes [209]. A second tankyrase, tankyrase-2 (PARP-6), was identified in 2001 by Lyons et al. [210]. The activity of the tankyrases is independent of DNA damage and the DNA repair processes [211]. The principal roles of tankyrase-1 are in regulating the length of telomeres, non-coding TTAGGG repeat regions at the end of chromosomes. Maintenance of telomere length during proliferation is an important feature of the immortality of malignant cell lines and inhibition of the enzyme directly responsible for telomere elongation, telomerase, was a goal in anticancer drug design for several years [212-214]. There is now much evidence that tankyrase-1 binds along the length of the telomeres and to telomere repeat binding factor 1 (TRF1) [215]. TRF1 acts to block access of telomerase to the telomere [216-218]. Tankyrase-1 ADP-ribosylates TRF1, forming unbranched oligomers of up to twenty ADP-ribose units; automodification also takes place [218-222]. The ADP-ribosylated TRF1 lifts off from the telomere, allowing telomerase access to the telomere to carry out its telomere-lengthening activity [218,223]. The loss of TRF1 from the telomere is an intrinsically reversible process, rendered irreversible by subsequent ubiquitination of the ADP-ribosylated TRF1, leading to degradation of the protein [219]. Thus tankyrase-1 acts to promote elongation of telomeres [219,224-228]. Inhibition of this PARP has been proposed as a target for drug design for cancer therapy [229]; this approach to preventing telomere elongation may not suffer from the long induction time seen with some telomerase inhibitors [230].

Tankyrase-2 is also associated with telomeres [230] and binds to tankyrase-1 and to TRF1 [231]; it may also fulfill similar roles to tankyrase-1 in telomere length regulation [218]. Overexpression of tankyrase-2 leads to cell death by necrosis [232].

Other roles have also been proposed for the tankyrases, making them not quite such a clear-cut target for drug design. For example, tankyrases have been found at centrosomes, nuclear pores and the Golgi apparatus [209,224]; at the Golgi, tankyrase-1 is known to bind to the insulin-responsive aminopeptidase IRAP [233] and tankyrase-2 has been implicated in vesicle trafficking [210]. Tankyrase-1 is also involved in the regulation of apoptosis in human myeloid leukaemia cells [234]. Thus therapeutic inhibition of the tankyrases may have effects other than simply causing shortening of telomeres during cell proliferation.

How can the tankyrases be inhibited, which small molecules inhibit the tankyrases and what are the observed effects of tankyrase inhibition? Little is known of the molecular structure of the tankyrases, except that their NAD+-binding domain has considerable sequence homology to PARP-1/PARP-2 and no crystal structures are available; thus no attempt to carry out rational design of selective tankyrase inhibitors has yet been reported. Disruption of the tankyrase-1 gene has been observed to cause mitotic arrest in some cell types [235]. 3-Aminobenzamide (3-AB) inhibits both tankyrase-1 and tankyrase-2 [231,236] but this compound is also the archetype of inhibitors of PARP-1 [94]; thus there are no known selective inhibitors. As with PARP-2, “the proof of the pudding will be in the eating”; we will not know what the effects of selective inhibition of the tankyrases will be until we have selective inhibitors.

Finally, PARP-7 has been tentatively identified as an independent form that is induced by tetrachlorodibenzodioxin (TCDD) [237] but its importance, structure and function are unknown; thus it cannot be considered as a realistic therapeutic target at present. However, this industrial toxin also selectively induces the cytochrome P450 isoform CYP1A1, which is involved in the carcinogenesis of polycyclic aromatic hydrocarbons; the mediators of the induction are also implicated in cellular response to stress, especially hypoxia [238,239]. It would be interesting to speculate as to whether or not there is any connection between PARP-7 and these processes.

No significant progress has yet been made on useful selective inhibitors of the PARP isoforms; all the inhibitors so far tested against PARP-1 and PARP-2 show very modest selectivity. However, since these two isoforms are both involved in control of DNA repair in response to damage by cytotoxic drugs and therapeutic ionising radiation, it may well be that selectivity turns out to be undesirable in a clinically useful inhibitor. However, the tankyrases present an interesting new target for drug design, in that inhibition may offer a new approach to modulating the telomere-regulating system in the light of the modest therapeutic success of telomerase inhibitors.

**POLY(ADP-RIbose)GLYCOHYDROLASE (PARG)**

Therapies targeting the metabolism of ADP-ribose polymers have primarily focused on PARP-1 inhibition. However, as with most other biochemical pathways, there
are more than one key regulatory enzyme involved and, hence, more than one possible point of therapeutic intervention. Poly(ADP-ribose)ylation is a dynamic process, as indicated by the very short half-life (less than 1 minute) [240] of the polymer in vivo. The transient nature of the polymer is largely due to its fast degradation by a major catabolic enzyme: poly(ADP-ribose) glycohydrolase (PARG, EC 3.2.1.143) [241]. PARG was first discovered by Miwa and Sugimura more than three decades ago [242] but, compared to its metabolic partner PARP-1, there was an apparent lack of research progress in this field. This is largely due to difficulties in purifying the enzyme and its low cellular abundance [243]. However, recent successful cloning and characterization of the cDNA encoding PARG have enabled detailed studies of this enzyme and its biological functions. It is now established that mammalian PARG is a 110 kDa protein consisting of a nuclear localization signal, a leucine zipper-like dimerisation domain and a 65 kDa C-terminal catalytic domain [244-246]. Its amino-acid sequence is highly conserved among mammals, with human, cow, rat and mouse sequences sharing more than 80% homology [243]. As opposed to PARP-1, it is present only in very small amounts in the cell and is largely localised within the cytoplasm [245]. The presence of a putative nuclear export signal possibly provides a mechanism for the shuttling of PARG from the cytoplasm to the nucleus where poly(ADP-ribose)ylation occurs [246].

The biological actions of PARP-1 and PARG are closely coordinated. Once the ADP-ribose polymer is synthesised by PARP-1, it is almost immediately hydrolysed by the constitutively active PARG, which cleaves the ribose-ribose linkage in the linear (1′′→2′′ glycosidic bond) and branched (1′′→2′′ glycosidic bond) portions of the polymer (see Scheme (1)) to produce ADP-ribose monomers [241]. Because the $K_m$ value of PARG is much lower for larger ADP-ribose polymers (<0.3 µM) than for smaller ones (10 µM), the enzyme probably hydrolyses bigger fragments first, then switches to removal of ADP-ribose units one by one [247]. This removal of ADP-ribose polymers from the autodigestion domain of PARP negates their down-regulatory effects on PARP-1 and frees the site for further polymer synthesis. Thus, the biosynthesis and degradation of ADP-ribose polymer constitute a cycle, the regulation of which is absolutely necessary for the normal growth of all poly(ADP-ribose) producing cells [88].

However, under condition of PARP-1 over-activation, such a rapid removal of the inhibitory ADP-ribose polymers by PARG would quickly reactivate PARP-1, allowing for a continuous turnover of poly(ADP-ribose), thereby further worsening NAD$^+$ and ATP shortage and markedly accelerate cell death [248]. It follows that an inhibition of PARG, under such circumstances, will have two beneficial effects: It indirectly inhibits PARP-1 by allowing the latter to resume negative feedback control on its own activity and it breaks the suicidal, energy-depleting cycle of poly(ADP-ribose) synthesis and degradation [249]. PARG may, therefore, be an alternative target for pharmacological intervention in diseases where PARP-1 inhibition is useful, such as ischaemia-reperfusion injury and inflammatory disorders.

Such realisation of the potential of PARG inhibition in the treatment of a wide range of diseases has stimulated considerable interest in the development of PARG inhibitors. Recent research has identified two families of compounds with encouraging PARG inhibitory properties in cell free assays. They are the ADP-ribose analogues [250], as exemplified by adenosine diphosphate (hydroxymethyl)pyrrolidinediolph (ADP-HPD, IC$_{50}$ 0.12 µM) and tannin derivatives [251,252], such as the ellagittannin oenothein B (IC$_{50}$ 4.8 µM), gallotannin (K, 25 µM), nobotannin (K, 4.8 µM) and 1,2,3,4,6-O-penta-β-D-galloylgucose (PGG, IC$_{50}$ 5.5 µM) (Fig. (9)).

ADP-HPD is a structural mimic of the oxonium intermediate which is presumably formed during the hydrolysis of NAD$^+$ [253]. It is the most specific and potent PARG inhibitor reported to date [250]. However, its high polarity and associated poor membrane permeability hinder further investigations in biological systems [254]. As such, most studies have focussed on the use of the relatively less potent tannins. Preliminary studies by different research groups have revealed promising cellular protective effects in various in vitro models of cell death. For instance, Ying and Swanson found that both gallotannin and nobotannin are effective in protecting mouse neurons and astrocytes against cell death induced by hydrogen peroxide, N-methyl-D-aspartate (NMDA) and N-methyl-1′′-nitro-N′′-nitrosoguanidine [255,256]. In addition, PGG (the basic unit in the tannin family) also protects P388D1 macrophage cell against hydrogen peroxide-induced cytotoxicity.

However, recent work by several independent research groups to re-investigate the effects of tannins on PARG has raised serious doubts about the efficacy of this class of inhibitor in intact cells. They noticed that gallotannin binds strongly and non-specifically, via the phenolic OH, to biomolecules, such as protein, albumin and gelatin. This could cause DNA strand breaks which, in turn, activates PARP-1, leading to an accumulation of cellular ADP-ribose polymers and an apparent PARG inhibition [257]. They also suggested that these phenolic groups could potentially react with the hydroxyl free radicals. Thus, it is possible that gallotannin protects against hydrogen peroxide-induced cell death by working as a hydrogen peroxide scavenger rather than as a PARG inhibitor [258,259]. This is supported by the observation that polyphenols without PARG inhibitory property also confer substantial protection against hydrogen peroxide-induced cell death, with quercetin being equally potent [260].

In view of these highly controversial experimental data and the many undesirable properties of these early PARG inhibitors, which preclude them as possible drug candidates, further development of PARG inhibitors with lower molecular weight, greater potency and specificity is clearly needed. This has resulted in an emergence of several families of second-generation PARG inhibitors, such as ethacridine (K, 7 µM) [261], an acridine derivative, and tilorone analogues; among which the activity of N,N-bis(3-phenylpropyl)-9-oxofluorene-2,7-diamide (GPI 16552, IC$_{50}$ 1.7 µM) is particularly noteworthy. This inhibitor has recently been demonstrated to significantly reduce the infarct volume in an in vivo model of cerebral ischaemia-reperfusion injury [262]. The need to accelerate the drug discovery process has also prompted pioneering work by Koh et al. to identify the inhibitor binding site of PARG...
and to examine the structural requirements for optimal binding to the active site [263,264]. Using ADP-HPD as the lead inhibitor, they found that both the ADP and the HPD portions of the molecule were required for binding to the PARG active site. In particular, the adenine ring is important for activity and may be involved with either a hydrophobic or a hydrogen-bonding interaction with the active site. Substitution at the 8-position with either bulky or lengthy groups resulted in a loss of PARG inhibition. The cis-hydroxyl groups on the pyrrolidine ring are also deemed crucial for activity as analogues lacking one or both hydroxyl functions have greatly diminished activity. Presumably, they help to anchor and orientate the substrate within the active site through hydrogen bond interactions.

Unquestionably, PARG inhibition has tremendous therapeutic potential. However, several important issues have also arisen recently with regards to the feasibility and safety of PARG inhibition. Unlike PARP-1 inhibition, an inhibition of PARG is not expected to prevent poly(ADP-ribosyl) synthesis, which is important for the maintenance of genomic integrity and DNA repair. Hence, theoretically, the use of this approach will have the added advantage of not interfering with the normal operation of these processes. However it was recently found that PARG inhibition does affect poly(ADP-ribosyl)ation-dependent transcription process. For instance, inhibition of PARG activity resulted in a drastic reduction in the expression of HMG proteins 14 and 17, both of which are important for the expression of several genes that are important for DNA repair [265]. Incidentally, Rapizzi et al. also observed that PARG inhibition activates poly(ADP-ribose)-dependent transcription of proinflammatory genes in macrophages, leading to a selective expression of inducible NO synthase and cyclooxygenase-2 [266].

In addition, PARG probably have other functions that are independent of PARP-1 and poly(ADP-ribosyl)ation. This was supported by a recent discovery that, in human cells undergoing apoptosis, PARG was cleaved at a relatively early stage by caspase-3, one of the main executioners of apoptosis, to two enzymatically active C-terminal fragments (85 kDa and 74 kDa) [267]. Therefore, a putative role for PARP-1 in the regulation of apoptosis has been suggested. Maruta et al. proposed that the mono(ADP)ribose liberated by PARG is eventually converted to ATP by ADP-ribose
pyrophosphorylase. Such a local accumulation of ATP around the damaged site could serve as a signal to activate apoptotic cell death, which is an energy-dependent process [268]. It is also noteworthy that while PARP-1 knockout mice showed no major untoward consequences in their development [168,269], PARG gene deleted cells appeared to have an increased sensitivity towards DNA damage [270]. A similar deletion in Drosophila also showed progressive neurodegeneration with a strong accumulation of poly(ADP-ribose), especially in the central nervous system [271]. These data, together with the fact that a viable PARG knockout mouse has yet to be successfully generated, could indicate that this enzyme is indispensable for normal cellular growth and function.

While de Murcia described the ever-growing PARP family as the "expanding universe of PARP proteins" [272], only one PARG enzyme has been reported thus far. Although it is possible that this enzyme alone is responsible for the degradation of poly(ADP-ribose) synthesised by the various PARP homologues, one cannot exclude the possibility that other mammalian PARGs may await to be discovered, especially with the recent characterisation of two cDNAs encoding proteins similar to PARG in Arabidopsis [273]. If this is true, what will be the biological consequences of inhibiting these PARG homologues? Also, what will be the effects of PARG inhibition on other members of the PARP family?

Answers to these exciting questions might lead to yet other promising areas of drug discovery. The future challenges of PARG research, therefore, lie in the development of more selective, potent and pharmacologically acceptable PARG inhibitors for animal testing in vivo, as well as the use of molecular biology tools to generate animal models that are deficient in the expression of PARG.

CONCLUSIONS

Five questions were posed at the outset of this review and a concluding response can now be given to each of them. Can water-solubility be designed into PARP inhibitors without loss of potency? The answer is clearly "yes", as has been demonstrated by the success of 5-AIQ and other inhibitors with tertiary amines incorporated into side-chains remote from the pharmacophore. Brain-penetrating PARP inhibitors have also been successfully designed. These advances bring pharmaceutical design into the medicinal chemistry of PARP inhibition. Some potential clinical applications require tissue-selective PARP-I inhibition; is this possible through prodrug approaches? Prodrugs of PARP inhibitors are in their infancy. Reductively triggered potential prodrugs have been developed which are very efficient in chemical systems; however, the real test for these will come in drug release experiments in hypoxic cells in vitro and in experimental solid tumours in vivo. Furthermore, since PARP inhibition is an anti-inflammatory event and inflamed synovium (in rheumatoid arthritic joints) is hypoxic, bioreductively activated prodrugs of PARP inhibitors may have applications in site-specific treatment for arthritis and other inflammatory diseases. Are there potential problems associated with chronic inhibition of the PARP-mediated DNA-repair process? At present, the problems are hypothetical, rather than actually observed, since there are no studies published on chronic inhibition of PARP on a timescale relevant to chronic treatment in humans. Current clinical trials are probably too short to address this issue and longer-term trials (for example, using PARP inhibition as part of an anti-inflammatory regimen) may need to address this issue. Is isoform-selectivity essential, desirable or even possible? The answer to this question is that we do not yet know. Inspired medicinal chemistry could lead to isoform-selective inhibitors, certainly for PARP-2 and possibly for the tankyrases and vPARP. When we have these selective inhibitors, the effects of selective inhibition can be defined more clearly and therapeutic applications can be identified. PARP activity can be inhibited by inhibition of PARG; will this be a viable strategy for future drug design? Inhibition of PARG leads to failure to remove the automodification of PARP-1 during the cycle, thus PARP-1 can only undergo one cycle under these circumstances and poly(ADP-ribosyl)ation (in response to DNA damage) will be suspended. This is an interesting new approach to modulating the system and a true appreciation of its value awaits the development of suitable small-molecule drugs with good pharmacological properties.

Inhibition of PARP, or of one or more PARPs, is an exciting prospect for treatment of a wide range of diseases [88]. PARP inhibitors have taken over thirty years to come to clinical trial, the true test of a new therapy, and the results of ongoing trials are eagerly awaited. Particularly in the areas of tissue-selective prodrugs and isoform-selective inhibitors, there are great opportunities for drug design and development for core targets in many diverse diseases.

REFERENCES
