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EXPRESSION IN YEAST AND PURIFICATION OF FUNCTIONAL RECOMBINANT HUMAN POLY(ADP-RIBOSE)POLYMERASE (PARP). COMPARATIVE PHARMACOLOGICAL PROFILE WITH THAT OF THE RAT ENZYME

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Human poly(ADP-ribose)polymerase (PARP) was expressed in the yeast line JEL1 under the control of a GAL promoter. Proteins were extracted and human recombinant PARP purified to apparent homogeneity. The pharmacological profile of this human enzyme was characterised in terms of the effects of known inhibitors of PARP belonging to various chemical families and this was compared with that of the rat enzyme purified from rat testes, using the same purification protocol. The rat and the human enzymes appeared very similar in terms of their sensitivities to those selected inhibitors.

Keywords: Poly(ADP-ribose)polymerase; Recombinant human enzyme; Rat enzyme; Relative sensitivity to inhibitors

Abbreviations: AEBSF, aminoethyl benzene sulfonyl fluoride; ADP, adenosine diphosphate; dpm, disintegrations per minute; DMSO, dimethyl sulfoxide; HT, hydroxyapatite; IC_{50}, 50%-inhibitory concentration; NAD, nicotinamide dinucleotide; PARP, poly(ADP-ribose)polymerase

INTRODUCTION

Poly(ADP-ribose)polymerase (PARP) is a 113 kD nuclear enzyme which catalyses the transfer of ADP-ribose moieties from nicotinamide dinucleotide

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(NAD) to itself and to several other nuclear proteins notably including histones, p53, topoisomerases I and II. PARP is activated up to 500-fold upon binding to DNA strand breaks (see reviews[1,2]). PARP is activated in response to DNA-damaging agents, such as alkylators and irradiation. Interestingly, PARP−/− knockout mice have been shown to be more sensitive than isogenic wild-type mice to the alkylating agent, N-methyl-N-nitrosourea, and to γ-ray irradiation.[3] Moreover, PARP interacts directly with the polymerase α-primase complex[4] and binds to a protein of the base excision repair pathway, XRCC1,[5] which in turn interacts with DNA ligase II and DNA polymerase β. Therefore, PARP may act as a DNA nick sensor, recruiting proteins of the base excision repair complex to the site of DNA damage and so favour access of this complex to DNA by decondensing chromosomes via ADP-ribosylation of histones.[6] In line with a role for PARP in DNA repair (see reviews[7–9]), several independant series of *in vitro* experiments have shown an enhancement of the cytotoxicity induced by alkylating agents in combination with, for example, either 1,3-bis(2-chloroethyl)-1-nitrosourea and 6-aminonicotinamide[10] or, more recently, temozolomide and a series of PARP inhibitors.[11] Similarly, enhancement of the cytoxicity of γ-irradiation has been reported in combination with PARP inhibitors by several groups, as reviewed earlier.[7] However, even with the more recent and more potent PARP inhibitors, these combinations only resulted in an enhancement factor of approximatively three, as judged in terms of respective IC_{50} values. The search for novel potent inhibitors of PARP requires screening for activity at the enzyme level and rat testes have been used as a convenient enzyme source. In order to rule out any potential differences in terms of sensitivities to these PARP inhibitors between the rat and human enzymes, in this study we have expressed native human PARP in yeast and purified it to apparent homogeneity. Then the pharmacological profile of both the rat testis and the human enzyme have been compared using known inhibitors belonging to structurally-different chemical classes, namely benzamide, phenantridinone, naphthalimide and quinazoline derivatives.[7,12]

MATERIALS AND METHODS

Materials

The compounds used in the present study were all solubilised in DMSO. The various PARP inhibitors were purchased from Sigma (Saint Quentin

Fallavier, France), with the exception of NU-1076 (2-(4-methoxyphenyl)-1H-benzimidazole-4-carboxamide) which was provided by Pierre Fabre Medicament (Castres, France). The plasmid YpADPRTGal,[13] encoding human PARP, was a generous gift from Dr. B. Auer, University of Innsbrück, Austria.

Production of Human PARP in Yeast

Colonies of the yeast strain JEL1, transformed with YpADPRTGal,[13] plated onto selective agar medium and grown at 30°C were resuspended in 100 ml medium (casaminoacids 2%, yeast nitrogen base 0.67%, glycerol 3%, lactic acid 1%, adenine 20 mg/l) plus 2% glucose and grown at 30°C. When the extinction, measured at 600 nm, reached a value of 1.2–1.5, yeast were transfered to 3–5 l of the same medium and permitted to grow until the extinction again reached a value of 1.2–1.5. Then these yeast cells were centrifuged for 5 min at 6000 rpm using a Beckman (Gagny, France) JA 14 rotor in a Beckman type J2 centrifuge at room temperature before resuspension in 3–5 l of medium containing 2% galactose followed by incubation for 48 h. After which yeast cells were harvested by centrifugation under the same conditions, resuspended in 500 ml water plus 0.2 mg/ml aminoethyl benzene sulfonyl fluoride (AEBSF) (Sigma). After a similar centrifugation, the yeast cells were resuspended in 50–100 ml of buffer B (100 mM Tris HCl pH 8.0, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 10% glycerol, 25 mM metabisulfite, 0.5 mg AEBSF/ml). The resultant pellet was weighed and resuspended in 1–2 volumes of buffer B and either used straightaway for purification or stored at −70°C. Yields of up to 8 g/l were obtained.

Purification of PARP

All procedures were carried out at 4°C. For the purification of human PARP from yeast, 5 ml glass beads (212–300 μM) were added to 8 ml yeast pellet in Corning glass tubes and yeast membranes were disrupted by vortexing 10 times for 30 s. For the purification of PARP from rat testis, after castration the testes were minced with a scalpel, mixed with one volume of buffer B′(buffer B plus 0.3 M KCl) and homogenized with a Potter homogeniser. Subsequent steps (modified from[14]) were common to both rat and human enzymes. Homogenates were centrifuged for 10 min at 15 000 rpm in a J 20.1 Beckmann rotor. The supernatant was filtered through a 0.8 μm Millipore (Saint Quentin Yvelines, France) filter in a syringe, and applied to a 5 ml Biorad (Ivry sur Seine, France) hydroxyapatite (HT) column

preequilibrated in buffer B′, at a flow rate of 1.5 ml/min. The HT column was washed sequentially with buffer B′, buffer B′ plus 300 mM phosphate, buffer B′ plus 500 mM phosphate.

PARP eluted in buffer B′ plus 300 mM phosphate. The HT eluate (600 mM in salt) was made 0.25 M with respect to salt with buffer B and applied to a 1 ml double strand DNA–cellulose column (Sigma) preequilibrated with buffer B plus 0.25 M KCl. The column was washed sequentially with the same buffer and then buffer B plus 1 M NaCl. PARP eluted with 1 M NaCl.

Determination of PARP Activity

As for the PARP assay itself,[14] 90 µl reaction mixture (calf thymus DNA 20 µg/ml, histone H1 20 µg/ml, NAD 10 µM, 0.02 µCi ^{3}H-NAD (NEN, Les Ulis, France), containing the amount of purified PARP corresponding to an activity of 5000 dpm/assay, in 80 mM Tris, 8 mM DTT, 10 mM $MgCl_2$, pH 8.0 were added to 10 µl of either drug or vehicle in an Eppendorf tube and incubated for 20 min at 25°C. The reaction was stopped by the addition of 100 µl ice cold 50% TCA. One hundred µl aliquots were distributed onto Whatman glass fibre filters and the radioactivity associated with histones estimated by scintillation counting. Assays were performed in duplicate on at least 3 separate occasions and results are expressed in terms of percent inhibition.

Miscellaneous Biochemical Procedures

Protein content was determined according to Bradford[15], separated by SDS-PAGE according to Laemmli[16] on a 10% polyacrylamide gel and either stained using a Silver Stain kit (Bio Rad, Ivry sur Seine, France) or transferred to a nitrocellulose sheet (Amersham, Les Ullis, France), according to Towbin *et al.*[17] for 2 h at 400 mA. All subsequent incubations were carried out at room temperature in blocking buffer (10% non-fat milk, 10% foetal calf serum in PBS). The nitrocellulose sheet was incubated in blocking buffer for 1 h before the addition of an anti-PARP antibody (Serotec, Oxford, UK) diluted 1/4000 and then left overnight. After extensive washing in PBS, incubation was performed in the presence of the secondary antibody, goat anti-mouse coupled to peroxidase (Jackson Immunoresearch Labs, West Grove, PA, USA) diluted 1/5000 for 1 h. PARP protein was visualised by enhanced chemiluminescence (ECL) according to the manufacturer's (Pierce, Rockford, IL, USA) instructions.

RESULTS AND DISCUSSION

Expression of Recombinant Human PARP in Yeast

Removal of glucose from the growth medium induced a blockage in the growth of YpADPRTGal JEL1 yeast, in either the presence or the absence of galactose. No regrowth was detected up to 72 h following glucose removal. This block though was not lethal, since yeast resumed growth upon addition of glucose. These results are in line with those reported by Kaiser *et al.*,[13] namely an inhibition of yeast growth correlated with PARP protein expression. The level of expression of the human PARP protein was followed by Western blotting as a function of time, following the addition of galactose. After 24 h in the presence of galactose, protein became detectable in yeast and the amount detected increased by 48 h (data not shown). Therefore, for producing large quantities of the human enzyme, the following strategy was adopted, namely growing yeast in the presence of glucose up to an extinction value of 1.2–1.5, pelleting the yeast down to resuspend it in a medium containing 2% galactose and harvesting the yeast 48 h later.

Purification of the PARP Enzyme

PARP from rat testis and a recombinant human enzyme expressed in yeast were purified to apparent homogeneity, as assessed by silver staining of a SDS-PAGE gel (Figure 1). The identity of the purified protein was assessed

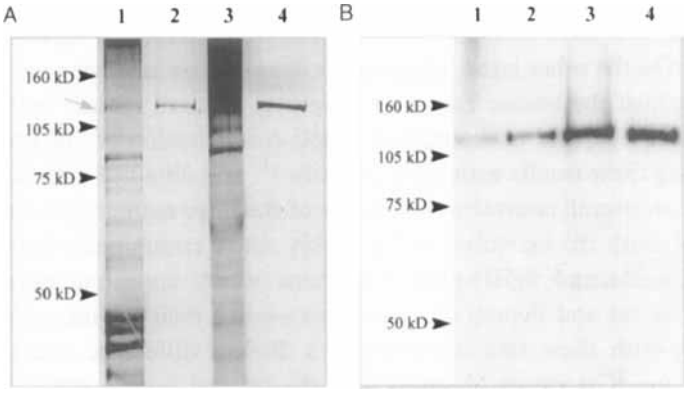

FIGURE 1 Assessment of the purity and identity of isolated human recombinant and rat testis PARP by separation on SDS-PAGE followed by silver staining (A) or immunoblotting (B). Lanes 1, 3: starting material (fractions before application to the HT column); lanes 2, 4: purified protein eluted from the DNA–cellulose column. Lanes 1, 2: human proteins; lanes 3, 4: rat proteins. Arrow: PARP protein; arrowheads: molecular mass standards.

TABLE I Enrichment in PARP specific activity during the purification process

Species	*Specific activity* (dpm/μg *protein*)		
	Starting material	*Eluate from HT*[a] *column*	*Eluate from DNA column*
Human	20	1700	120000
Rat	10	1800	250000

[a]HT: hydroxyapatite.

by Western blotting. Similar levels of activity, around 1700–1800 dpm/μg protein were obtained for both enzymes after the HT column step. Comparable activities around 120000 and 250000 dpm/μg protein respectively for the purified rat and human enzymes were obtained (Table I), although accurate determination was made difficult by the inaccuracy in assessing low amounts of proteins in the DNA–cellulose column eluates.

Sensitivity Profile of Rat and Human PARP Enzymes to a Panel of Inhibitors

Practically superimposable patterns of inhibition of the two enzymes were obtained with known PARP inhibitors, belonging to distinct chemical classes (Figure 2), such as 4-amino-1,8-naphthalimide, 2-nitro-6(5H)-phenantridinone, benzamide and NU-1076. IC_{50} values obtained with certain of these compounds, namely 3-aminobenzamide, benzamide, 2-nitro-6(5H)-phenantridinone and 4-hydroxyquinazoline (Table II) differed by a factor of less than 2, whilst those obtained with compounds such as 4-amino-1,8-naphthalimide, NU-1076 and 6(5H)-phenantridinone differed by factors of 3–6, at most. On the other hand, compounds described as inactive or marginally active against the bovine enzyme[17] were also inactive against both the rat and human enzymes at the highest tested concentration of 100 μM. When comparing these results with those published[17] and obtained with the bovine enzyme, an overall equivalent sensitivity of the three mammalian enzymes is apparent, with the exception of the highly active compounds 4-amino-1,8-naphthalimide and 6(5H)-phenantridinone which appeared more potent against the rat and human enzymes than against their bovine counterpart. Notably, with these two compounds, a 20-fold difference was observed between the IC_{50} values obtained with the rat and bovine enzymes. However, since the assay conditions and, notably the substrate concentrations, were not identical, being 10 μM NAD in the present study and 200 μM NAD in that of Banasik's,[12] such differences should not be overinterpreted. Indeed, the IC_{50} value obtained with NU-1076 against the purified human

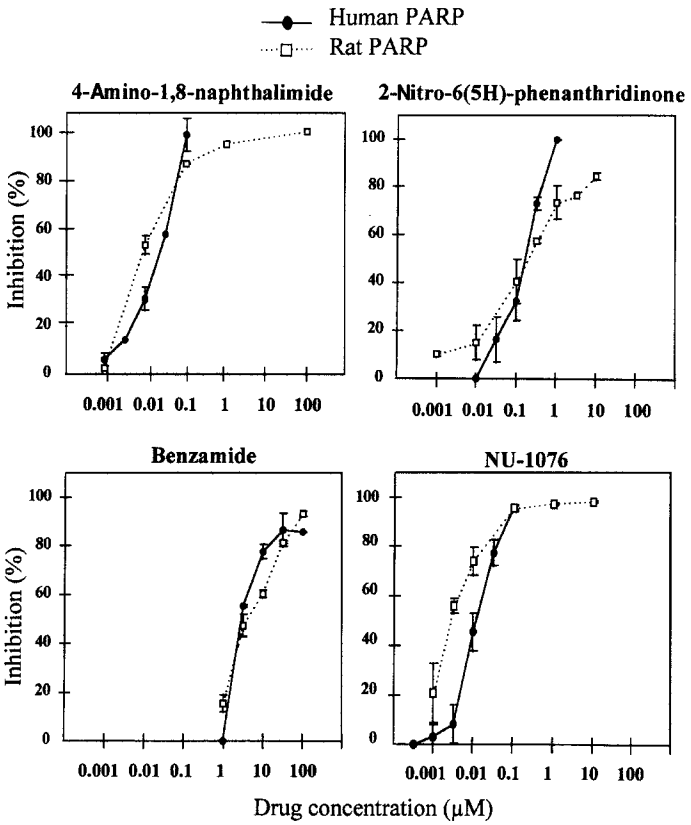

FIGURE 2 Effects of known inhibibitors on the activities of PARP from either rat or human origin. Results are expressed as a percentage of inhibition (±sem) as a function of the concentration of the inhibitor in the assay.

TABLE II Comparative pharmacological profile of the various mammalian PARP enzymes

Test compound[a]	*Human enzyme* IC_{50}[b] *values* (M)	*Rat enzyme* IC_{50}[b] *values* (M)	*Bovine enzyme* IC_{50}[b] *values from Ref.* [12] (M)
4-Amino-1,8-naphthalimide	2.3×10^{-8}	8.7×10^{-9}	1.8×10^{-7}
3-Aminobenzamide	2.0×10^{-5}	2.0×10^{-5}	3.3×10^{-5}
6(5H)-Phenantridinone	8.2×10^{-8}	1.4×10^{-8}	3.0×10^{-7}
4-Hydroxyquinazoline	1.0×10^{-5}	5.6×10^{-6}	9.5×10^{-6}
Benzamide	2.8×10^{-6}	3.9×10^{-6}	2.2×10^{-5}
2-Nitro-6(5H)-phenanthridinone	1.7×10^{-7}	1.8×10^{-7}	3.5×10^{-7}
NU-1076	1.2×10^{-8}	2.6×10^{-9}	nr[c]
4-Aminobenzamide	$> 1.0 \times 10^{-4}$	$> 1.0 \times 10^{-4}$	1.8×10^{-3}
Benzoic acid	$> 1.0 \times 10^{-4}$	$> 1.0 \times 10^{-4}$	nr[c]
3-Aminobenzamidine	$> 1.0 \times 10^{-4}$	$> 1.0 \times 10^{-4}$	nr[c]

[a]The highest concentration tested was 1.0×10^{-4} M, due to limitations of solubility. [b]IC_{50}: 50% inhibitory concentration. [c]nr: not reported.

enzyme (12 nM) was comparable to that reported for PARP in permeabilised murine leukemia cells (60 nM).[18]

Although PARP is considered to be a highly conserved protein, with an overall conservation of 62% including a 100% homology of a 50 amino acid block in the catalytic domain of the protein amongst vertebrate (human, mouse, bovine, chicken and *Xenopus*),[6,19] to our knowledge no comparative sensitivities of these mammalian enzymes to a panel of known inhibitors has yet been published. This issue is clearly of importance when considering establishing screening procedures for use in detecting new inhibitors of the human PARP enzyme.

The overall conclusion from this study therefore is that these rat and human enzymes demonstrated comparable sensitivities to the panel of inhibitors tested, whilst results published in the literature seem to indicate a lower sensitivity of the bovine enzyme to certain test compounds.

Therefore, these results support the validity of using the readily available rat enzyme, rather than the bovine one for initial screening of potential PARP inhibitors. Moreover, it has been shown here that the recombinant untagged human PARP enzyme can be expressed in yeast in amounts high enough to permit purification and characterisation of its sensitivity pattern to a panel of known inhibitors and that it can be considered for use in the screening of novel compounds.

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