An Improved Nonisotopic Test to Screen a Large Series of New Inhibitor Molecules of Poly(ADP-ribose) Polymerase Activity for Therapeutic Applications

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ABSTRACT
A reliable ELISA for screening large numbers of poly-(ADP-ribose) polymerase (PARP) inhibitors is described. The test is based upon the drop in PARP activity estimated by the decrease in poly(ADP-ribose) synthesis in the presence of inhibitor. This ELISA is easy to perform, rapid, and specific. It is extremely sensitive because a clear inhibition of the total reaction could be visualized with molecules used in the nanomolar range. The assay uses no radioactivity, and automation is possible with robots for large-scale investigations. This test is of great interest for the screening of chemical libraries and the discovery of new inhibitors (and possibly activators) of PARP. Such molecules have important applications in all abnormal situations involving DNA damage and oxidative stress, such as cancer, autoimmunity, diabetes, myocardial dysfunctions, certain infections, aging, and radiation/chemical exposure.

INTRODUCTION
PARP (E.C. 2.4.2.30) is a zinc-finger DNA-binding protein that detects and signals DNA strand breaks generated directly by genotoxic agents (monofunctional alkylating agents, oxygen radicals, and ionizing radiation) or indirectly during DNA repair during the first 6 h after DNA damage (6). Moreover, mouse embryonic fibroblasts derived from PARP-deficient mice performed very limited DNA repair during the first 6 h after DNA damage by alkylating agents (7).

To avoid futile DNA repair cycles during the execution phase of apoptosis, PARP is specifically cleaved and inactivated by several caspases after consensus sequence 211-DEVD-214 (8) located in the bipartite nuclear location signal (9), thus generating two fragments of M 85,000 and M 25,000. Caspase-3 (CPP32/Yama/apopain) is the most efficient processing enzyme for PARP (10, 11). Detection of PARP cleavage fragments provides a sensitive test for apoptosis (12). Because apoptosis occurs in a variety of cell types during the physiological organogenesis and maturation of the immune system and plays a crucial role during normal tissue homeostasis, manipulation of PARP activity under the control of particular inhibitors may have dramatic physiological consequences. Defects in apoptosis have been reported to be a cause of systemic autoimmunity (13, 14). Drugs with a direct or indirect role in apoptosis may thus have an important interest in the treatment of certain autoimmune diseases. The potentiation of cytotoxicity of both monofunctional alkylating agents and ionizing radiation by PARP inhibitors (15) has been confirmed by genetic and molecular studies (16, 17), thus demonstrating their potential to improve chemotherapy and radiotherapy strategies. Conversely, in certain situations, activator drugs could stimulate DNA repair and provide chemoprevention of cellular oxidative stress (18).

By attenuating the fall of intracellular NAD+ and ATP, inhibitors of PARP activity have recently been shown to reduce...
the infarct size caused by ischemia and reperfusion of the heart or skeletal muscle in rabbits (19). The same effect was obtained by the absence of PARP in PARP knockout mice (20, 21). Enhancer/inhibitor molecules of PARP activity may thus have a considerable interest in all situations involving DNA damage and oxidative stress such as inflammation, cancer, autoimmunity, diabetes, myocardial dysfunctions, certain infections (e.g., AIDS), aging, and radiation/chemical exposure (18, 22, 23).

A number of PARP competitive inhibitors have been designed and tested during the last decade (for a review, see Ref. 24). Among these inhibitors, benzamide and its derivatives 3-AB and 3-MB were found to be the most potent. However, these compounds caused significant side effects when used in animals (25). The search for new compounds requires screening tests that are specific, sensitive, rapid, and easy to handle. Tests that are presently available for such studies generally use a radioactive substrate (e.g., $[^{32}\text{P}]/\text{NAD}$), are performed on membrane (which is not convenient when testing a large series of compounds), and are time-consuming (e.g., see Refs. 26 and 27). Here we describe a simple ELISA for testing large numbers of inhibitors (and possibly activators) of PARP. The test presents all of the advantages of ELISA: it is reliable, reproducible, sensitive, and rapid. It can be rendered quantitative, and automation is possible with robots for large-scale investigations.
PBS-T and H2O2, the final reaction was visualized by adding anti-poly(ADP-ribose) MAb. After a 1-h incubation at 37°C and three washings with PBS-T, peroxidase-labeled goat antirabbit IgG (data not shown). Because one of the interests of such a test is to evaluate libraries of compounds in which an active molecule can be present in minute amounts, we sought an ELISA that could detect as low a concentration as possible. Depending on the activity of each inhibitor, a clear inhibition (≥30%) of the total reaction could be visualized with the molecules used in the nanomolar range. For example, an inhibition of 51% of the reaction (SD = 4.2 when the results from independent ELISAs were compared) was observed in the presence of 600 nM 3-MB; 40 nM NU1025 inhibited the reaction by 56%, and 8 nM of this inhibitor still reduced PARP activity by 23%.

RESULTS AND DISCUSSION

The ELISA assay displayed in Fig. 1 is based upon the drop in PARP activity evaluated by the decrease of poly(ADP-ribose) synthesis. Briefly, rPARP diluted in Tris-HCl buffer (pH 8) containing MgCl2 and ZnCl2 was first immobilized on a microtiter plate (Falcon; catalogue number 3912). After three washings with PBS-T, 1.25 μg/ml nicked DNA prepared as described previously (29) and diluted in the Tris buffer described above containing 50 μM β-NAD+ (Boehringer Manheim; catalogue number 127965) and 1 μM freshly prepared DTT (reaction medium) was added for 1 h at 4°C (200 μl/well). After three washings with PBS-T, the synthesis of poly(ADP-ribose) by activated PARP was detected by adding the anti-poly(ADP-ribose) MAb 10H (a gift from Dr. T. Sugimura, National Cancer Center Research Institute, Tokyo, Japan; IgG3, κ; Ref. 30) in culture supernatant diluted 1:1600 in PBS-T containing 0.4% BSA (200 μl/well). After a 1-h incubation at 37°C and three washings with PBS-T, peroxidase-labeled goat antimouse IgG3 (Nordic) diluted 1:5000 in PBS-T (200 μl/well) was added for 30 min at 37°C. After a final series of washings (twice with PBS-T and once with H2O2), a positive reaction was visualized by 3,3′,5,5′-tetramethyl-benzidine in the presence of H2O2 for 15 min at 37°C (150 μl/well). The reaction was stopped by adding 50 μl of HCl (final concentration, 0.25 M), and absorbance was measured at 450 nm. The incubation time of each reagent and the respective concentration of each reagent in the test were determined to obtain an optimal signal:noise ratio and a satisfactory sensitivity. The average intraplate coefficients of variation of absorbance values (measured as absorbance values) was significantly decreased, thus reducing the sensitivity of the test.

The average intraplate coefficients of variation of absorbance values without inhibitor were about 13% using 60 test wells/plate and 20% using 96 test wells/plate for mean absorbance values.

MATERIALS AND METHODS

The newly developed test to screen PARP inhibitors consists of the following steps (Fig. 1). Human rPARP (Ref. 28; 400 ng/ml) in Tris buffer [50 mM Tris-HCl (pH 8) containing 20 μM ZnCl2 and 4 mM MgCl2 (200 μl/well)] was immobilized on a polyvinyl chloride microtiter plate (Falcon; catalogue number 3912). After three washings with PBS-T, 1.25 μg/ml nicked DNA prepared as described previously (29) and diluted in the Tris buffer described above containing 50 μM β-NAD+ (Boehringer Manheim; catalogue number 127965) and 1 μM freshly prepared DTT (reaction medium) was added for 1 h at 4°C (200 μl/well).

To study potential PARP inhibitors, the compounds to be tested were included in the reaction medium (Fig. 1). The final reaction with and without inhibitor was compared.

When histone H1 was coated on the ELISA plates as a heterologous acceptor in addition to PARP that plays the role of the homologous acceptor of poly(ADP-ribose), the final reaction (measured as absorbance values) was significantly decreased, thus reducing the sensitivity of the test.

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<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Present studya</th>
<th>Rankin et al. (31)</th>
<th>Banasik et al. (32)</th>
<th>Boulton et al. (33)</th>
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<td>Benzamide</td>
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<td>NT</td>
</tr>
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<td>31,000</td>
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</table>

a The test conditions are described in the text and the legend of Fig. 3.
b NT, nontested; see the legend of Fig. 3 for the origin of products.

Table 1 Comparison of the IC50 values of the PARP inhibitors estimated by different procedures

The incubation time of each reagent and the respective concentration of each reagent in the test were determined to obtain an optimal signal:noise ratio and a satisfactory sensitivity. The efficiency of various inhibitors of PARP activity is illustrated in Fig. 3. Table 1 lists the IC50 values evaluated for classical PARP inhibitors. Similar results were obtained with two individual preparations of PARP.

The specificity of the assay was evidenced by several controls: no reaction was found in the absence of activated DNA, DTT, or NAD+ or when α-NAD+ (Sigma, N6754) was introduced instead of β-NAD+. The incubation time of each reagent and the respective concentration of each reagent in the test were determined to obtain an optimal signal:noise ratio and a satisfactory sensitivity. The level of poly(ADP-ribose) was very low when MgCl2 and ZnCl2 were omitted from the Tris buffer. The antibody 10H showed no reaction with rPARP and DNA. MAb 10H could be replaced with the same efficacy by a rabbit anti-poly(ADP-ribose) antiserum (a gift from Dr Y. Kanai, National Cancer Center Research Institute, Tokyo, Japan; Ref. 34). In this case, the final reaction was revealed using peroxidase-labeled goat antirabbit IgG (data not shown).
ance values corresponding to 1.37 (SD 0.18) and 1.26 (SD 0.26), respectively. The coefficients of variation of absorbance values between two different plates was ≤5.4% (mean absorbance values, 1.36 versus 1.26/96 test wells), and the intertest coefficient of variation of absorbance values was ≤1.5% (mean absorbance values, 1.28 versus 1.36/96 test wells).

In summary, this ELISA is easy to perform, rapid, specific, and extremely sensitive, and it uses no radioactivity. It is particularly suited to the study of a large series of molecules or a mixture of molecules designed to inhibit (and possibly to enhance) the activity of PARP.

REFERENCES

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