Poly(ADP-ribose) Polymerase-1 Inhibitor 3-Aminobenzamide Enhances Apoptosis Induction by Platinum Complexes in Cisplatin-Resistant Tumor Cells

Paul A. Nguewa[a], Miguel A. Fuertes[b], Victoria Cepeda[b], Carlos Alonso[b], Celia Quevedo[c], Manuel Soto[b] and José M. Pérez[c,*]

[a]*Departamento de Parasitología, Facultad de Farmacia, Universidad de La Laguna, Tenerife, Spain;* [b]*Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM), Facultad de Ciencias, Universidad Autónoma de Madrid, 28049-Madrid, Spain;* [c]*Departamento de Química Inorgánica, Facultad de Ciencias, Universidad Autónoma de Madrid, 28048-Madrid, Spain*

Abstract: Cisplatin is one of the most widely used antitumor drugs. However, as all the anticancer drugs currently used in clinic, cisplatin shows the phenomenon of drug resistance (intrinsic or acquired) against a wide variety of tumors. Poly (ADP-ribose) polymerase-1 is an enzyme involved in DNA repair and apoptotic cell death, which may be inhibited to increase cisplatin chemosensitivity of tumor cells so that cisplatin resistance may be circumvented.

In the present study we report that PARP-1 inhibitor 3-aminobenzamide (3-AB) increases the cytotoxic activity of the platinum compounds cisplatin, *trans*-[$PtCl_2$(4-picoline)(piperazine)] and transplatin against CH1cisR cisplatin-resistant ovarian tumor cells. In fact, a concentration of 3-AB of 1 mM not only increases the cytotoxic activity of these platinum complexes but also switches the mode of cell death from necrosis to apoptosis. Altogether, these data suggest that pharmacological modulation of PARP-1 by inhibitors may be a suitable strategy to fight against tumor resistance to platinum drugs.

Key Words: PARP-1 inhibitors, 3-aminobenzamide, platinum drugs, apoptosis, cancer chemotherapy.

1. INTRODUCTION

Cisplatin, [*cis*-diamminedichloroplatinum(II)] or *cis*-DDP, is highly effective in the treatment of testicular and ovarian cancers and is also employed for treating bladder, cervical, head and neck, oesophageal and small cell lung cancer [1]. The isomer of cisplatin, [*trans*-diamminedichloroplatinum(II)], (*trans*-DDP or transplatin), is devoid of antitumor efficacy [2]. As reported for all the currently known antitumor drugs, *cis*-DDP also shows the phenomenon of drug resistance (intrinsic or acquired) against a wide variety of tumors. At the molecular level, resistance to cisplatin is generally multifactorial and may be due to reduced drug accumulation within the cell, inactivation by thiol-containing biomolecules, increased repair/tolerance of platinum-DNA adducts, and defective apoptotic processes [3]. One strategy to circumvent cisplatin resistance is the design of platinum complexes that specifically deal with some or even all of the above-mentioned resistance mechanisms [4]. However, after more than 30 years of intensive research since the discovery of the antitumor properties of cisplatin, only 4 platinum drugs (cisplatin, carboplatin, oxaliplatin and nedaplatin) are currently registered for clinical use. Moreover, only one of these platinum drugs, oxaliplatin, is able to overcome cisplatin resistance [5]. An alternative strategy to fight against cisplatin resistance is the use of modulators of cisplatin chemosensitivity. So far, an increasing number of drugs and targets for modulation of cisplatin chemosensitivity has been discovered [6].

Poly(ADP-ribose) polymerases (PARPs enzymes), also known as poly(ADP-ribose) synthetases and poly(ADP-ribose) transferases, constitute a family of cell signalling enzymes (PARP-1, -2 and -3, Vault-PARP and Tankyrase-1,- 2 and -3) present in eukaryotes, which catalyze poly (ADP-ribosyl)ation of DNA-binding proteins [7-9]. These enzymes have emerged as critical regulatory components of the immediate cellular response to DNA damage. Poly(ADP-ribose) polymerase-1 (PARP-1), is the first characterized and the best known member of the PARP family. PARP-1, encoded by the ADPRT gene, is an abundant and highly conserved chromatin bound enzyme which binds to nicked DNA as a homodimer (MW= 2 x 113 kDa) and mediates protection against DNA damage. As shown in Fig. (**1**), upon binding to DNA breaks induced by *cis*-DDP, activated PARP-1 cleaves its substrate NAD^+ into nicotinamide and ADP-ribose moieties (ADPR) and polymerizes the latter through surface accessible glutamate residues onto nuclear acceptor proteins, and PARP-1 itself [10]. When DNA is mildly damaged, PARP-1 is activated and participates in the DNA repair process so that the cell survives. However, in the case of extensive DNA damage, PARP-1 is overactivated and induces a depletion of cellular NAD^+ and ATP levels leading to necrotic cell death [10]. Due to the dual response of PARP-1 to DNA damage and its involvement in cell death, pharmacological modulation of PARP-1 activity may constitute a useful tool to increase the activity of DNA-

*Address correspondence to this author at the Departmento de Química Inorgánica, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049-Madrid, Spain; Tel: +34914978454; Fax: 34913274457;
E-mail: josema.perez@uam.es

1573-4064/06 $50.00+.00

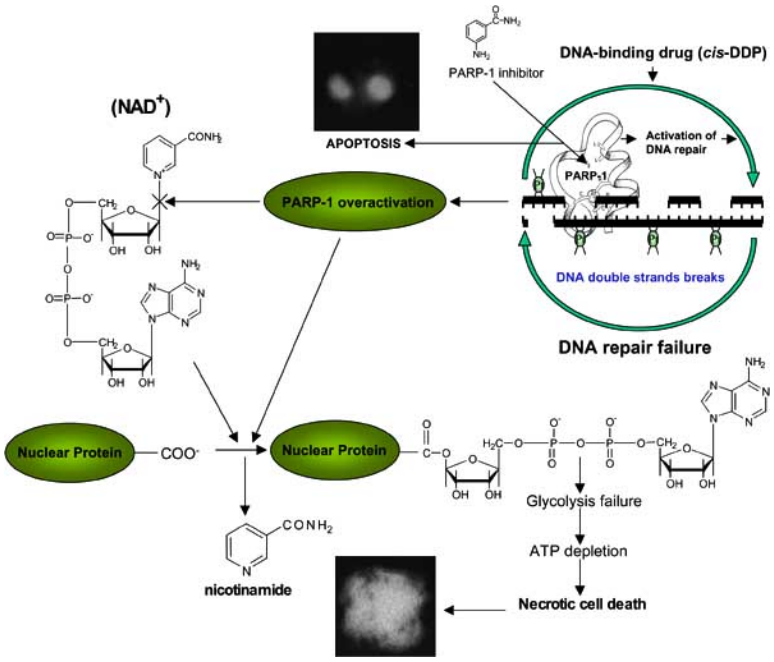

Fig. (1). PARP-1 overactivation leads to necrotic cell death and PARP-1 inhibition by compounds such as 3-aminobenzamide (3-AB) ends up in apoptosis.

binding antitumor drugs. In fact, various adjuvant strategies directed to modulate PARP-1 activity including the use of PARP-1 inhibitors [9] or the use of ATP-depleting agents [10] have been reported in recent years. Recent observations indicate that cells may be protected from necrosis by inhibition or inactivation of PARP-1 [11]. So, inhibition of PARP-1 activity in cells exposed to DNA-damaging drugs such as cisplatin would decrease DNA repair and would induce apoptotic cell death, decreasing necrotic cell death and preventing the pathological side effects of necrosis. In fact, it has been reported that PARP-1 inhibitors 3-aminobenzamide (3-AB) or NU1025 increase apoptosis and reduce necrosis induced by the DNA minor groove binder $MeOSO_2(CH_2)_2$-lexitropsin (Me-Lex) [12]. Most PARP-1 inhibitors have been designed to imitate the substrate-enzyme interactions of NAD^+ with the catalytic site of PARP-1 [7, 13]. Because of their structural resemblance to the substrate, these compounds are able to act as competitive inhibitors, blocking NAD^+ binding to the catalytic domain of PARP-1 enzyme. A common structural characteristic of PARP-1 inhibitors is either the presence of a carboxamide or an imide group built in a polyaromatic heterocyclic skeleton or a carbamoyl group attached to an aromatic ring [11]. The oxygen atom from the carbonyl group seems to function as a hydrogen acceptor, and the hydrogen atom from the amide or imide groups acts as a proton donor in the hydrogen-bond interaction with PARP-1.

In this paper we report that 3-aminobenzamide (3-AB) increases the cytotoxic activity of platinum compounds Fig. (**2**) against CH1cisR cisplatin-resistant ovarian tumor cells. Our results show that 3-AB not only increases the cytotoxic activity of platinum complexes but also switches the mode of cell death from necrosis to apoptosis.

2. RESULTS

2.1. Cytotoxic Activity of Platinum Compounds in the Pair of Tumor Cells CH1/CH1cisR

The cytotoxic activity of *cis*-DDP, *trans*-DDP and the new *trans*-platinum compound *trans*-[$PtCl_2$(4-picoline)(piperazine)] [14] was evaluated in the pair of human ovarian tumor cell lines CH1/CH1cisR. CH1 cells are sensitive to cisplatin while CH1cisR cells are resistant to cisplatin

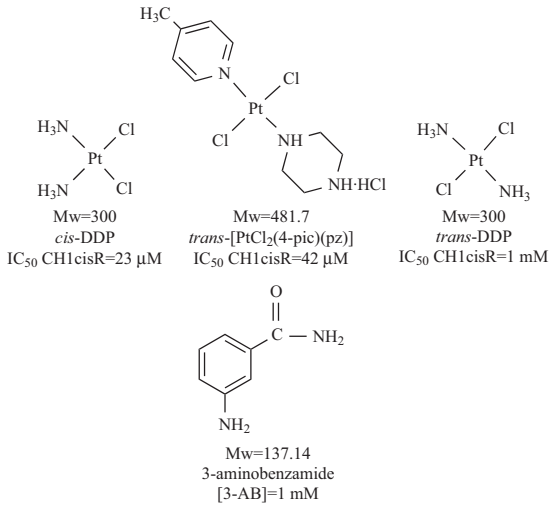

Fig. (2). Structures of platinum complexes: *cis*-DDP, *trans*-[$PtCl_2$(4-picoline)(piperazine)], and *trans*-DDP and of PARP-1 inhibitor 3-aminobenzamide (3-AB).

through enhanced DNA repair [15].The platinum drugs were incubated for 24 hours at 37ºC with CH1 or CH1cisR cells. Cell survival in control untreated cultures and in cultures treated with the platinum drugs was calculated by the MTT method [16]. The results of both the IC_{50} and resistant factors (RF) values are shown in Table **1**. IC_{50} is defined as the drug concentration that produces 50% of cell death. RF is defined as the ratio between the IC_{50}s of the cisplatin-resistant (CH1cisR) and the cisplatin-sensitive (CH1) cell lines. After 24 hours of drug treatment, *cis*-DDP showed a cytotoxic potency against CH1 cells which was 2.7-times and 36.7-times higher than those of *trans*-[$PtCl_2$(4-picoline)(piperazine)] and *trans*-DDP, respectively (IC_{50} values of 6 μM, 16 μM and 220 μM, respectively). Similarly, *cis*-DDP showed a cytotoxic potency against CH1cisR cells which was 1.8-times and 43.5-times higher than those of *trans*-[$PtCl_2$(4-picoline)(piperazine)] and *trans*-DDP, respectively (IC_{50} values of 23 μM, 42 μM and 1000 μM, respectively). Interestingly, however, the RF value of *trans*-[$PtCl_2$(4-picoline)(piperazine)] (RF = 2.6) was lower than those of *cis*-DDP (RF = 3.8) and *trans*-DDP (RF = 4.5) suggesting that this new *trans*-Pt(II) compound is able to partially circumvent cisplatin resistance in CH1cisR cells. In fact, it is known that in this pair of cell lines RF values ≤ 2 are indicative of circumvention of cisplatin resistance [17].

2.2. 3-AB Switches Platinum-Induced Tumor Cell Death from Necrosis to Apoptosis

We used annexin-V/propidium iodide (PI) flow cytometry to detect the mode of cell death induced in CH1cisR cells by the platinum compounds either alone or in combination with 3-AB. Annexin-V binds phosphatidyl serine residues, which are asymmetrically distributed towards the inner plasma membrane but migrate to the outer plasma membrane during apoptosis. On the other hand, PI is a DNA intercalator that enters the cell membrane and goes to the nucleus when the cell membrane integrity has been lost [15]. Therefore, in a typical annexin V-PI scattergram, the annexinV^-/PI^- cell population (left bottom quadrant) contains alive cells, the annexinV^+/PI^- cell population (right bottom quadrant) contains early apoptotic cells, the annexinV^+/PI^+ cell population (right upper quadrant) contains late apoptotic cells, and finally, the annexinV^-/PI^+ cell population (left upper quadrant) contains necrotic cells.

We first identified a concentration of 3-AB, which is safe and does not induce killing of CH1cisR cells. So, Fig. (**3B**) shows that when CH1cisR cells are incubated for 24 h at 37ºC with 1mM of 3-AB all the population of CH1cisR cells is located in the left bottom quadrant of alive cells as observed in control untreated CH1cisR cells Fig. (**3A**).

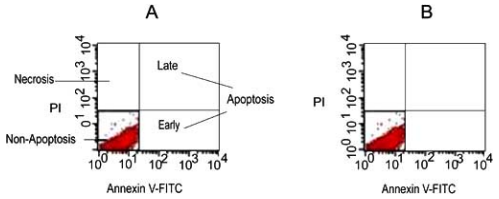

Fig. (3). Representative annexin-V/PI fluorescence scattergrams showing: (A) control untreated CH1cisR cells and (B) CH1cisR cells treated for 24 h with 1 mM 3-AB. The data were obtained from four independent experiments.

Fig. (**4A**) shows that the IC_{50} of *cis*-DDP (23μM) induced a 25% of early apoptosis (right bottom quadrant), a 10% of late apoptosis (right upper quadrant) and a 17% of necrosis (left upper quadrant). Interestingly enough, Fig. (**4B**) shows that when CH1cisR cells were incubated with a combination of *cis*-DDP (IC_{50} = 23 μM) plus 3-AB (1mM), the percentage of cells in early apoptosis increased to 61% (right bottom quadrant) and that of cells in late apoptosis increased to 33% (right upper quadrant). However, no necrotic cells were observed (upper left quadrant). Moreover, the percentage of alive CH1cisR cells decreased to 6% (left bottom quadrant) indicating that 1 mM of 3-AB increases the cytotoxicity of *cis*-DDP from the IC_{50} to approximately the IC_{95}.

Similar results were observed with *trans*-[$PtCl_2$(4-picoline)(piperazine)] and *trans*-DDP in combination with 3-AB. However, in the case of *trans*-DDP, 3-AB induced an increase of cytotoxicity (from IC_{50} to IC_{85}), which was slightly lower than those observed for *cis*-DDP and *trans*-[$PtCl_2$(4-picoline)(piperazine)] (from IC_{50} to IC_{95}). So, it may be observed in Figs. (**4C** and **4D**) that the combination of the IC_{50} of *trans*-[$PtCl_2$(4-picoline)(piperazine)] (42 μM) + 3-AB (1mM) increased the percentage of early apoptotic CH1cisR cells from 28.8% to 61.3%. In the same way, the percentage of late apoptotic cells increased from 15.5% to 32.9%. In addition, as previously observed for *cis*-DDP, the cytotoxic effect of *trans*-[$PtCl_2$(4-picoline)(piperazine)] increased from the IC_{50} to the IC_{95} and no necrotic cells were detected. Figs. (**4E** and **4F**) show that the combination of the

Table 1. IC_{50} Values (μM) and Resistance Factors (RFs) in the Pair of Ovarian Tumor Cell Lines CH1/CH1cisR After 24 Hours of Incubation with the Platinum Compounds

	CH1 (IC_{50})	CH1cisR* (IC_{50})	RF (IC_{50} CH1cisR/IC_{50} CH1)
trans-[$PtCl_2$(4-pic)(pz)]	16 ± 2	42 ± 3	2.6
cis-DDP	6 ± 1	23 ± 3	3.8
trans-DDP	220 ± 11	1000 ± 50	4.5

*CH1cisR cells are resistant to cisplatin through enhanced DNA repair of platinum-DNA adducts.

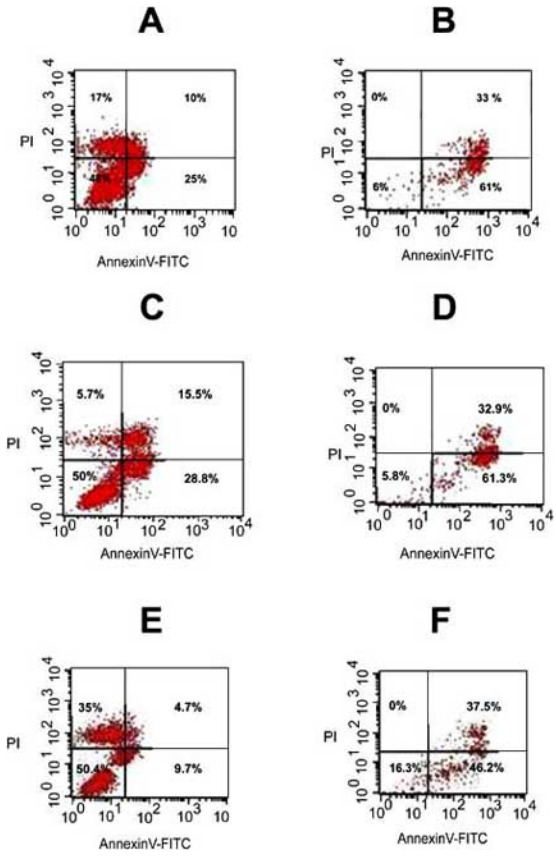

Fig. (4). Quantification of apoptosis and necrosis in CH1cisR cells after 24 h exposure to: (A) IC_{50} of *cis*-DDP. (B) 1 mM 3-AB + IC_{50} of *cis*-DDP. (C) IC_{50} of *trans*-[$PtCl_2$(4-picoline)(piperazine)]. (D) 1 mM 3-AB + IC_{50} of *trans*-[$PtCl_2$(4-picoline) (piperazine)]. (E) IC_{50} of *trans*-DDP and (F) 1 mM 3-AB + IC_{50} of *trans*-DDP. Representative annexin-V/PI fluorescence scattergrams from four independent experiments.

IC_{50} (1mM) of *trans*-DDP plus 1 mM of 3-AB also increased the percentage of early apoptotic cells from 9.7% to 46.2% and that of late apoptotic cells from 4.7% to 37.5%. Moreover, the combination of *trans*-DDP + 3-AB decreased the percentage of CH1cisR undergoing necrosis from 35% to 0%. However, 3-AB produced a lower increase in the cytotoxic effect of *trans*-DDP, which went from the IC_{50} to the IC_{85}.

We carried out similar studies with the protozoan parasite *Leishmania infantum,* which is the causative agent of a spectrum of diseases called Leishmaniases [18]. *L. infantum* is a kinetoplastid parasite, which lacks PARP-1 enzyme so that in the event of DNA damage PARP-1 can not be activated [18]. Fig. (**5**) shows the results obtained when the protein content of cellular extracts of CH1cisR cells (lane 2), *Trypanosome cruzi* (lane 2, used as a control) and *Leishmania infantum* (lane 3) are subjected to polyacrylamide gel electrophoresis in denaturing conditions (SDS-PAGE) and subsequent western blot analysis to detect the presence of PARP enzymes using polyclonal antibodies against PARP

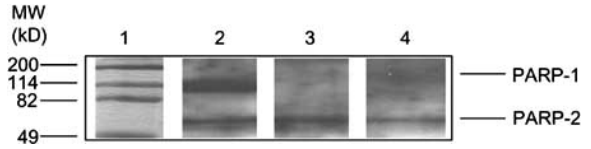

Fig. (5). Western blot autoradiography showing anti-PARP reactivity of protein extracts from different cellular lysates. Lane 1: MW marker (kD), lane 2: CH1cisR cells, lane 3: *Tripanosome cruzi* cells and lane 4: *Leishmania infantum* cells. Prior to western blotting analysis, protein extracts were subjected to SDS-PAGE. The lines indicate the MWs corresponding to PARP-1 monomers (114 kD) and PARP-2 monomers (62 kD).

catalytic fragment (catPARP). As may be observed in lane 2, two protein bands were detected in CH1cisR extracts. A first band with a molecular weight (MW) of 114 kD and a second one with a MW of 62 kD. By comparison with lane 1 (protein MW marker), these bands should correspond to PARP-1 and PARP-2 monomers, respectively [7]. However, in lanes 3 (*T. cruzy* extract) and 4 (*L. infantum* extract) only one protein band with a MW of 62 kD corresponding to PARP-2 monomers was detected. So, as expected, these data shows that in contrast with higher organisms, Trypanosomatids (*T. cruzi* and *L. infantum*) do not possess PARP-1 enzyme.

cis-DDP alone (IC_{50}= 150 μM) or *cis*-DDP(150 μM) + benzamide (1mM) were incubated with *L. infantum* promastigotes for 72 h and the induction of either programmed cell death (PCD) or necrosis was determined by annexin-V/propidium iodide (PI) flow cytometry. As expected, Figs. (**6A** and **6B**) show that the combination of *cis*-DDP plus 3-AB did not have any effect neither in the cytotoxicity of *cis*-DDP alone nor in the percentages of apoptotic or necrotic *L. infantum* promastigotes.

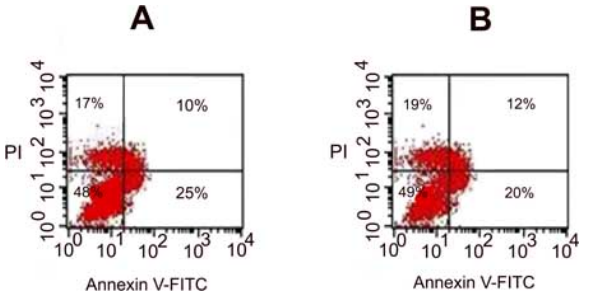

Fig. (6). Quantification of apoptosis and necrosis in promastigotes of *Leishmania infantum*. (A) control untreated promastigotes and (B) promastigotes treated for 72 h with the IC_{50} of *cis*-DDP. Representative annexin-V/PI fluorescence scattergrams from four independent experiments.

Altogether these result suggest that in CH1cisR cells, PARP-1 inhibition by 3- AB is able to increase the cytotoxic activity of the platinum drugs so that the mode of cell death switches from necrosis to apoptosis. In contrast, in *L. infantum* promastigotes, which lack PARP-1 activity, 3-AB has no effect on the cytotoxic activity of the platinum compounds.

2.3. 3-AB Does Not Induce Cell Cycle Alteration in CH1cisR Cells

We wanted to know whether the differences in cytotoxic activity between the platinum drugs alone or in combination with 3-AB were correlated with specific cytotoxic effects of 3-AB on the cell cycle phases. So, we perform cell cycle analysis by flow cytometry after PI staining of CH1cisR cells incubated for 24 hours with the IC_{50} values of the Pt complexes alone, 3-AB alone or a combination of the IC_{50}s of the platinum drugs with 1 mM of benzamide. Table **2** shows the percentages of CH1cisR cells in the different phases (G1, S and G2/M), which were obtained in this set of experiments. It may be observed that 1 mM 3-AB does not have any effect in the subpopulations of CH1cisR cells in each of the cell cycle phase. On the other hand, *cis*-DDP kills CH1cisR cells mainly in S and G2/M phases because the percentages of cells in S and G2/M decreases from 25.5% and 16.5% in control cells to 15.7% and 7.5% in *cis*-DDP-treated cells, respectively. However, *cis*-DDP does not kill cells in G1 phase. However, *trans*-DDP kills CH1cisR cells mainly in G1 and G2/M while *trans*-[Pt(4-pic)(pz)] kills CH1cisR cells mainly in G1 and S. Interestingly, Table **2** shows that 3-AB increased the cytotoxicity of the platinum drugs but did not affect the distribution of CH1cisR cells in the different cell cycle phases.

3. DISCUSSION

After more than three decades of intensive research since the initial discovery of the antitumor properties of cisplatin, only one of the four platinum complexes licensed for clinical use (oxaliplatin) is able to circumvent cisplatin resistance [19]. An alternative strategy to the design of new platinum drugs capable of circumventing *cis*-DDP resistance is the use of inhibitors of PARP-1 enzyme in combination with cisplatin and the other platinum marketed drugs (i.e., carboplatin, nedaplatin and oxaliplatin) [8].

We have reported in this paper an *in vitro* study in which we have found that PARP-1 inhibitor 3-AB is able to increase the cytotoxic activity of the platinum compounds *cis*-DDP, *trans*-DDP and *trans*-[$PtCl_2$(4-picoline)(piperazine)] in CH1cisR human ovarian tumor cells. It should be pointed out that when these platinum drugs are used in combination with 3-AB no necrotic CH1cisR cells are observed. So, these data indicate that in the subpopulation of CH1cisR cells in which PARP-1 is overactivated due to extensive platinum damage to DNA, inhibition of PARP-1 by 3-AB avoids NAD^+ and ATP depletion and subsequent necrosis so that the cells die through apoptosis. That 3-AB must be inhibiting PARP-1 activity in CH1cisR cells may be deduced from the fact that in the single-celled parasite *Leishmania infantum* (which lacks PARP-1) the combination of 3-AB + *cis*-DDP is unable to increase either the cytotoxicity or the amount of apoptotic cells relative to cisplatin alone.

Nicotinamide (effective inhibitory concentration, EC_{50} = 22 μM) and the structurally related 3-AB (EC_{50} = 33 μM) were identified as competitive PARP-1 inhibitors in the 1970´s [20]. The analysis of the mechanism of action of these compounds has revealed that the carboxamide group is critical in their inhibitory activity [9]. In addition, it has been reported that benzamides are able to bind to DNA nicks, avoiding their recognition by PARP-1, and therefore preventing the activation of the enzyme [21]. Therefore, in CH1cisR (which are resistant to cisplatin through enhanced repair of Pt-DNA adducts), 3-AB should inhibit PARP-1 binding to DNA with subsequent prevention of both DNA repair and ATP depletion, which leads to induction of apoptotic cell death by the platinum drugs.

Our data show that when the platinum compounds are administered alone, the higher the IC_{50} value (e.g., *trans*-DDP) the higher the subpopulation of CH1cisR cells, which die through necrosis. However, an interesting finding from our results is that biologically inactive *trans*-DDP increases its cytotoxic activity in the presence of 3-AB so that all CH1cisR cells die through apoptosis.

The PARP-1 inhibitor most frequently used *in vivo* chemo-potentiation studies is 3-AB because of its lack of

Table 2. Percentages of CH1cisR Cells in the Different Phases (G1, S and G2/M) of the Cell Cycle After 24 Hours of Treatment with the IC_{50}s of the Platinum Compounds or After 24 Hours of Treatment with the IC_{50}s of the Platinum Compounds + 1mM 3-AB

	G1 (%)	S (%)	G2/M (%)	Cell Death (%)
CH1cisR control cells	53.0	25.5	16.5	5.0
3-AB	54.0	24.5	17.0	4.5
cis-DDP	26.3	15.7	7.5	50.5
cis-DDP+3-AB	2.5	1.5	0.5	95.5
trans-DDP	14.5	19.3	10.7	45.5
trans-DDP+3-AB	4.6	6.0	3.4	86.0
trans-Pt[(4-pic)(pz)]	17.6	15.5	15.9	51.0
trans-Pt[(4-pic)(pz)]+3-AB	1.8	1.6	1.6	95.0

toxicity, even at high doses [9]. Enhancement of *in vivo* activity against The Ehrlich ascites tumor and other cancer models using 3-AB has been reported for bleomycin [22], cisplatin [23], chlorambucil [24] and cyclophosphamide [25]. The optimum dose of 3-AB is ≈ 500 mg/kg/injection. However, the use of higher doses of 3-AB complicate the interpretation of the data, because of the hypothermic effects of such doses, which, for instance, reduce the plasma clearance of the antitumor test drug [24]. So, 3-AB exhibits some drawbacks as a PARP-1 inhibitor for potential application in the clinical setting that must be solved.

In summary, the use as adjuvant chemotherapeutics of benzamide analogues (e.g., 3-AB) as well as other chemical classes of PARP-1 inhibitors such as dihydroisoquinolinones [26] and new tetracyclic heterocyclic compounds [24] may constitute a suitable strategy to increase the activity and circumvent resistance of tumors to platinum antitumor drugs.

4. EXPERIMENTAL SECTION

4.1. Chemicals

cis-DDP, *trans*-DDP and 3-AB were purchased from Sigma-Aldrich, Alcobendas (Madrid), Spain. *trans*-[$PtCl_2$(4-picoline)(piperazine)] was prepared as previously reported [14]. Stock solutions of the platinum compounds or 3-AB were freshly prepared before use at a final concentration of 1 mg/ml in double distilled water.

4.2. Cell Culture and Cytotoxicity of the Platinum Compounds

CH1 and CH1cisR cell lines were cultured in a humidified 5% CO_2 atmosphere at 37 ºC with DMEM medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO Spain Ltd). Cell survival in compound-treated cultures was analyzed by using a system based on the tetrazolium compound 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), which is reduced by living cells to yield a soluble formazan product that can be assayed colorimetrically [16]. Cells, in exponential phase of growth ($2x10^5$ cells/ml), were plated in 96-well sterile plates, at a density of 10^4 cells/well in 100 µl of medium and were incubated with the compound dissolved in DMEM at concentrations of 1, 5, 25, 50,100, 200, 500, 1000 and 1500 µM in a volume of 100 µl/well. After 24 hours of incubation, 50 µl of a freshly diluted MTT solution was added at a concentration of 1 mg/ml into each well and the plates were incubated for 5h at 37°C in a humidified 5% CO_2 atmosphere. Cell survival was evaluated by measuring the absorbance at 520 nm, using a Whittaker Microplate Reader 2001. Cytotoxic activity was expressed as IC_{50} values (compound concentration that induces a 50% of cell death). IC_{50} values were calculated from curves constructed by plotting promastigotes survival (%) versus compound concentration (µM). All data were obtained from four independent experiments.

4.3. Flow Cytometric Analysis

4.3.1. Quantification of Apoptosis by Annexin V Binding

Exponentially growing CH1cisR cells or *L. infantum* promastigotes were exposed to the IC_{50} of the platinum drugs, to 1mM benzamide or to a combination of 3-AB (1 mM) + IC_{50} of the platinum drugs for 24h or 72h, respectively. Subsequently, the cells were resuspended in annexin-V binding buffer (BD Biosciences PharMingen, San Diego, CA). Propidium iodide (PI, 2.5 µl; Sigma) and 1 µg/ml of annexin V-fluorescein isothiocyanate (BD Biosciences PharMingen) were added, and the cells were left at room temperature before flow cytometric analysis in a FACScalibur apparatus (BD Biosciences, San Jose, CA). The percentage of apoptotic cells induced (percentage of annexin V-positive/PI negative cells) was calculated from the annexinV/PI scattergrams [27]. The data were obtained from four independent experiments with duplicates.

4.3.2. Cell Cycle and Hypodiploidy Analysis

Relative DNA content (hypodiploidy) was assessed using propidium iodide (PI; Sigma) after cell permeabilization with 70% methanol (Merck) in PBS as previously reported [28]. Cells were analyzed using a FACScan flow cytometer.

4.4. Statistical Analysis for the Cytotoxicity Data

Where appropriate, statistical significance was tested using a Student's test (two-tailed, unpaired). A P value of <0.05 was considered significant [15].

4.5. Polyacrilamide Gel Electrophoresis (PAGE) and Western Blot Analysis

CH1cisR, *T. cruzy* and *L. infantum* cells were suspended in lysis buffer [50 mM tris(hydroxymethyl)aminomethane, Tris.HCl, pH 8.0, containing 1mM EDTA (ethylenediaminetetraacetate), 100 mM NaCl and 0.5 mM phenylmethylsulfonylfluoride (PMSF, Sigma Co.)]. Proteins from the cell extracts were subjected to SDS-PAGE and subsequent blot transfer as previously reported [29]. Nitrocellulose filters from the blot transfers were incubated for 24 h at room temperature with a (1/1000) dilution of polyclonal antibodies (Santacruz biotech) against PARP catalytic domain (catPARP), which is highly conserved among the different PARP enzymes [7]. Then, the filters were incubated with a (1/2000) dilution in PBS-$Tween_{20}$ of goat antirabbit peroxidase-conjugated polyclonal antibody (Nordick laboratories) for 1 h at room temperature. The specific binding was revealed by ECL western blotting detection reagents (Amershan-Pharmacia biotech).

ACKNOWLEDGEMENTS

This work was supported by Spanish Ministerio de Educación y Ciencia (SAF 2004-03111) and in part supported by Spanish Fondo de Investigaciones Sanitarias (Grant C03/04). We also thank sponsorship by European COST Action D20/0003/00. An institutional grant from Fundación Ramón Areces is also acknowledged. VC and CQ are the recipients of predoctoral (FPI) and postdoctoral (Juan de la Cierva) fellowships from the Spanish Ministerio de Educación y Ciencia, respectively.

ABBREVIATIONS

3-AB = 3-aminobenzamide

ADPRT = ADP-ribosyltransferase

DMEM = Dulbecco's modified Eagles medium

EC_{50} = Effective inhibitory concentration, which induces 50% of enzyme inhibition

EDTA = Ethylenediaminetetraacetate

MW = Molecular weight

PARP = Poly(ADP-ribose) polymerase

PBS = Phosphate-buffered saline (1M phosphate buffer, pH 7.2, and 0,1 M NaCl)

PI = Propidium iodide

PMSF = Phenylmethylsulfonylfluoride

RF = Resistance factor, which is defined as the ratio between the IC_{50}s of the resistant and the sensitive cell lines

SDS-PAGE = Sodium dodecyl sulphate-polyacrilamide gel electrophoresis

Tris = Tris(hydroxymethyl)aminomethane

REFERENCES

[1] Fuertes, M.A.; Castilla, J.; Alonso, C.; Perez, J.M. *Curr. Med. Chem.* **2003**, *10*, 257.
[2] Perez, J.M.; Fuertes, M.A.; Alonso, C.; Navarro-Ranninger, C. *Crit. Rev. Oncol. Hematol.* **2000**, *35*, 109.
[3] Gonzalez, V.M.; Fuertes, M.A.; Alonso, C.; Perez, J.M. *Mol. Pharmacol.* **2001**, *59*, 657.
[4] Fuertes, M.A.; Castilla, J.; Alonso, C.; Perez, J.M. *Curr. Med. Chem. Anti-Canc. Agents* **2002**, *2*, 539.
[5] Fuertes, M.A.; Castilla, J.; Nguewa, P.A.; Alonso, C.; Pérez, J.M. *Med. Chem. Rev.-Online* **2004**, *1*, 187.
[6] Fuertes, M.A.; Alonso, C.; Perez, J.M. *Chem. Rev.* **2003**, *103*, 645.
[7] Nguewa, P.A.; Fuertes, M.A.; Valladares, B.; Alonso, C.; Perez, J.M. *Prog. Biophys. Mol. Biol.* **2005**, *88*, 143.
[8] Nguewa, P.A.; Fuertes, M.A.; Alonso, C.; Perez, J.M. *Mol. Pharmacol.* **2003**, *64*, 1007.
[9] Southan, G.J.; Szabo, C. *Curr. Med. Chem.* **2003**, *10*, 321.
[10] Martin, D.S.; Bertino, J.R.; Koutcher, J.A. *Cancer Res.* **2000**, *60*, 6776.
[11] Virag, L.; Szabo, C. *Pharmacol. Rev.* **2002**, *54*, 375.
[12] Tentori, L.; Balduzzi, A.; Portarena, I.; Levati, L.; Vernole, P.; Gold, B.; Bonmassar, E.; Graziani, G. *Cell Death Differ.* **2001**, *8*, 817.
[13] White, A.W.; Almassy, R.; Calvert, A.H.; Curtin, N.J.; Griffin, R.J.; Hostomsky, Z.; Maegley, K.; Newell, D.R.; Srinivasan, S.; Golding, B.T. *J. Med. Chem.* **2000**, *43*, 4084.
[14] Nguewa, P.A.; Fuertes, M.A.; Iborra, S.; Najajreh, Y.; Gibson, D.; Martinez, E.; Alonso, C.; Perez, J.M. *J. Inorg. Biochem.* **2005**, *99*, 727.
[15] Perez, J.M.; Kelland, L.R.; Montero, E.I.; Boxall, F.E.; Fuertes, M.A.; Alonso, C.; Navarro-Ranninger, C. *Mol. Pharmacol.* **2003**, *63*, 933.
[16] Alley, M.C.; Scudiero, D.A.; Monks, A.; Hursey, M.L.; Czerwinski, M.J.; Fine, D.L.; Abbott, B.J.; Mayo, J.G.; Shoemaker, R.H.; Boyd, M.R. *Cancer Res.* **1988**, *48*, 589.
[17] Najajreh, Y.; Perez, J.M.; Navarro-Ranninger, C.; Gibson, D. *J. Med. Chem.* **2002**, *45*, 5189.
[18] Nguewa, P.A.; Fuertes, M.A.; Valladares, B.; Alonso, C.; Perez, J.M. *Trends Parasitol.* **2004**, *20*, 375.
[19] Wang, D.; Lippard, S.J. *Nat. Rev. Drug Discov.* **2005**, *4*, 307.
[20] Banasik, M.; Komura, H.; Shimoyama, M.; Ueda, K. *J. Biol. Chem.* **1992**, *267*, 1569.
[21] McLick, J.; Hakam, A.; Bauer, P.I.; Kun, E.; Zacharias, D.E.; Glusker, J.P. *Biochim. Biophys. Acta* **1987**, *909*, 71.
[22] Kato, T.; Suzumura, Y.; Fukushima, M. *Anticancer Res.* **1988**, *8*, 239.
[23] Chen, G.; Pan, Q.C. *Cancer Chemother. Pharmacol.* **1988**, *22*, 303.
[24] Cosi, C. *Exp. Opin. Ther. Patents* **2002**, *12*, 1047.
[25] Petrou, C.; Mourelatos, D.; Mioglou, E.; Dozi-Vassiliades, J.; Catsoulacos, P. *Teratog. Carcinog. Mutagen.* **1990**, *10*, 321.
[26] Suto, M.J.; Turner, W.R.; Arundel-Suto, C.M.; Werbel, L.M.; Sebolt-Leopold, J.S. *Anticancer. Drug Des.* **1991**, *6*, 107.
[27] Montero, E.I.; Perez, J.M.; Schwartz, A.; Fuertes, M.A.; Malinge, J.M.; Alonso, C.; Leng, M.; Navarro-Ranninger, C. *Chembiochem* **2002**, *3*, 61.
[28] Soto, M.; Iborra, S.; Quijada, L.; Folgueira, C.; Alonso, C.; Requena, J.M. *Biochem. J.* **2004**, *379*, 617.
[29] Fuertes, M.A.; Perez, J.M.; Soto, M.; Lopez, M.C.; Alonso, C. *J. Biol. Inorg. Chem.* **2001**, *6*, 107.

Received: 22 July, 2005 **Revised: 06 September** **Accepted: 13 September, 2005**