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Poly(ADPR)polymerase inhibition and apoptosis induction in cDDP-treated human carcinoma cell lines

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ABSTRACT

Poly(ADPR)polymerases' (PARPs) inhibitors potentiate the cytotoxic effects of chemotherapeutic agents like alkylating compounds and TOPO I poisons, while their action in combination with cisplatin still needs investigation. In fact, one of the earliest responses to DNA single- or double-strand breaks is the synthesis of poly(ADP-ribose) (PAR) by PARPs; these enzymes are components of DNA repair machineries and substrates of caspases. Cisplatin (cDDP) yields intra- and inter-strand DNA cross-links and several proteins that recognise cDDP-induced DNA damage, such as p53, are also targets of poly(ADP-ribosyl)ation. We compared the effects of treatments with cDDP and the PARPs inhibitor PJ34 in p53 mutated carcinoma cell lines (HeLa, KB, HT29) that exhibited differential sensitivities to the drugs, in terms of cell growth inhibition and onset of apoptosis. In cDDP-resistant HT29 cells we determined: (i) PJ34 potentiation of cDDP-induced cell growth inhibition; (ii) an increment of PARP-1 automodification following cDDP treatment. In cDDP-sensitive HeLa cells, we found that the drug induced apoptotic cell death associated with caspase-dependent PARP-1 proteolysis.

1. Introduction

Due to the involvement of poly(ADPR)polymerases (PARP) in the repair of DNA damage induced by certain anticancer agents and or radiation, PARP inhibitors are under investigation as chemo- and radiosensitizers for cancer treatment.

The PARP family of enzymes [1,2] catalyse a post-translational protein modification reaction that can occur immediately after exposure of cells to DNA damaging agents [3]. *In vivo*, 90% of ADP-ribose polymers (PAR) deriving by the use of β-NAD^+ substrate, are attached to the auto-modification domain of PARP-1 (EC 2.4.2.30), the main enzyme catalysing this reaction. Furthermore, PARP-1 has been found to covalently poly(ADP-ribosyl)ate a number of nuclear components (hetero-modification) including both structural and functional proteins [1]. Moreover, PAR chains (up to 200 residues long) linked to PARP-1 are able to interact with several target proteins containing a ''polymer binding motif'' [4] thereby modulating their function; for instance, p53 specific binding to its DNA consensus sequence can be regulated by such a mechanism. Poly(ADPR)glycohydrolase (PARG) is the PARPs' enzymatic counterpart catalysing PAR catabolism [1,5].

The intervention of PARP-1 and PARP-2 takes places at early steps of the DNA repair process as these enzymes bind to, and are activated by DNA nicks [4]. Upon poly(ADP-ribosyl)ation, PARPs recruit to the damaged site components of BER and NER machineries [6,7]. Therefore, inhibition of PARP activity would prevent repair enzymes recruitment to DNA breaks, hampering strand rejoining and consequent generation of permanent single/double strand breaks, which, in turn, trigger the apoptotic process.

PARP-1 is well known as a ''death substrate''. This role of PARP is based on the fact that it was one of the first identified substrates of caspases (cysteinyl aspartate-specific proteases),

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the main executioners of apoptosis [8]. During apoptosis caspase-7 and caspase-3 cleave PARP-1 into two fragments, p89 and p24, thus suppressing PARP activity.

Then, according to a ''double-edge sword'' role [9]: (1) PARP-1 activated by mild to moderate genotoxic stimuli facilitates DNA repair by signaling cell cycle arrest and by interacting with DNA repair enzymes. As a result, DNA damage is repaired and cells survive without the risk of passing on mutated genes: (2) more severe DNA damage induces apoptotic cell death during which caspases inactivate PARP-1; this pathway allows cells with unrepairable DNA damage to become eliminated in a safe way. Cleavage of PARP avoids hyperactivation of PARP by the ensuing DNA fragmentation, thereby preventing cells from the pathological sequelae of necrosis a less controlled mechanism posing danger for bystander cells.

The above-described role of PARP in the cell death is essential in order to plan PARP inhibition to enhance cytotoxicity, depending on the nature and severity of DNA damage. Moreover, chemotherapy-induced apoptosis can be considered a suitable strategy [10,11].

The potential of PARP inhibitors to increase the chemotherapeutic index has led to the development of a wide range of specific inhibitors – quinazolinone derivates – like NU1025 or PJ34, which display increased potency compared to the prototype 3-amino-benzamide (3-ABA) [12].

Previous studies demonstrated that PARP inhibition is a suitable strategy to enhance the efficacy of methylating agents as temozolomide (TMZ) [13], however PARP inhibitors did not sensitize MMR-deficient cells that were resistant to cisplatin [14]. Moreover, we showed that PARP-1 inhibition is also able to increase cytotoxicity of DNA topoisomerase I poisons such as the camptothecin analogue topotecan (TPT) [15].

At present we afford the analysis of carcinoma cells treated with cisplatin (cDDP) in combination with PJ34 PARP(s) inhibitor. Platinum compounds are among the most widely used and effective anticancer drugs. These agents interact with DNA generating preferentially intra-strand cross-links that, if not removed by the nucleotide excision repair (NER), are highly cytotoxic [16].

It is well known that the p53 status might exert a role in determining the cellular sensitivity to a number of therapeutic agents, including cisplatin, although conflicting results have been observed in various cancers [17]. Since mutations of p53 are usually associated with drug resistance, we analysed human carcinoma cell lines with a mutated p53 protein.

In regard to the use of PARP inhibitors as enhancer of platinum compounds efficacy, previous results were obtained with the PARP inhibitor CEP-6800 in a model of non-small cell lung carcinoma Calu-6 [18]. However, the Calu-6 cell line is extremely sensitive to cisplatin and the entity of chemopotentiation, even though statistically significant, is modest.

On the light of the all of these findings we investigated the potential of the use of PJ34 as adjuvant of cDDP in resistant human colon carcinoma cells HT29^{p53mut}, compared to cervical carcinoma HeLa cells and epidermoid carcinoma KB cells, that in spite of their p53 mutation appeared to be cDDP-sensitive. Moreover, we investigated the molecular mechanism underlying such an effect, in terms of modulation of PARP-1 activity.

2. Materials and methods

2.1. Cell culture and reagents

The human carcinoma cell lines HeLa, KB and HT29 were maintained in Dulbecco's modified Eagle's medium (DMEM; CAMBREx Profarmaco, Italy) containing 10% (v/v) heat-inactivated foetal bovine serum (FBS; BIOWHITTAKER Inc. USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM L-glutamine (Cambrex Profarmaco, Italy) and incubated at 37 °C in a humidified atmosphere, *plus* 5% CO_2.

Cisplatin (cDDP) was from SIGMA, Italy, and PJ34 from ALEXIS Biochemicals was supplied by Vinci-Biochem, Italy. The cocktail of protease inhibitors was from ROCHE-Diagnostic, Italy. Nicotinamide adenine [adenylate-^{32}P] dinucleotide-[^{32}P]-NAD$^+$ (~1000 Ci/mmole, 10 mCi/ml) and [^{3}H]NAD$^+$ nicotinamide (nicotinamide 3,5,8[^{3}H] adenine dinucleotide 250 mCi/mmole) were supplied by GE HEALTHCARE, Italy.

PVDF (poly-vinylidene-fluoride) membrane was from MILLIPORE Corporation, USA.

Anti-PAR mouse monoclonal antibody (H-10), anti-PARP-1 polyclonal antibody from ALEXIS Biochemicals were supplied by Vinci-Biochem, Italy. Anti-p53 (DO-1, sc-126), anti-AIF (H300, sc-5586) and anti-α-actin (H300, sc10731) rabbit polyclonal antibodies, as well as goat anti-mouse and goat anti-rabbit IgG HRP-conjugate, from SANTA-CRUZ Biotechnology Inc. were supplied by DBA Italy.

All other chemicals were of the highest quality commercially available.

2.2. Cell growth inhibition

HeLa and HT29 cells were seeded in 10 mm plates at 2.5×10^3 cells/. After 24 h, either Hela and HT29 previously mentioned cell cultures were treated with different concentrations of cDDP (10–50 μM) and PJ34 (0.1–10 μM) and cell growth inhibition was assessed at different time points (12, 24, 48, 72 and 96 h) using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.

All the experiments were performed in triplicate.

2.3. Cytofluorimetric analysis

Control and treated cells were detached by enzymatic treatment (Trypsin/EDTA 0.02%), washed in PBS$^{w/o\,Ca^{++}/Mg^{++}}$ pooled with floating cells and recovered by centrifugation at 150 × *g* for 15 min at 4 °C. Cells were fixed in 70% ethanol and stored at −20 °C until analysis.

After a washing in PBS$^{w/o\,Ca^{++}-Mg^{++}}$, 1×10^6 cells were stained in PI staining solution (25 μg/ml of PI, 0.05 mg/ml of RNAse in PBS$^{w/o\,Ca^{++}-Mg^{++}}$, pH 7.4) overnight at 4 °C and DNA-flow cytometry was performed in duplicate by a FACScalibur flow cytometer (BECTON DICKINSON) coupled with a CICLOPS work station (Cytomation). Cell cycle analysis was performed by the ModFit LT software (VERITY SOFTWARE HOUSE Inc.). For each sample 15,000 events were stored in list mode file.

2.4. Isolation of nuclear and post-nuclear fractions

To isolate sub-cellular fractions, cells were suspended in a buffer containing 30 mM Tris–HCl pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1% (v/v) Triton-X100, 20% glycerol, 2 mM PMSF and the protease inhibitors cocktail solution. After 30 min of incubation on ice, cellular suspensions were centrifuged at 800 × *g* for 30 min at 4 °C and the nuclear fractions recovered in the pellet. The supernatant represents the post-nuclear fraction.

Nuclear fractions were resuspended in 20 mM HEPES pH 7.8, containing 0.2 mM EDTA, 1.5 mM $MgCl_2$, 20% glycerol and the protease inhibitors cocktail solution. Protein concentration was determined using the Bradford protein assay reagent (BIO-RAD) with bovine serum albumin as a standard.

2.5. Detection of protein poly(ADP-ribosylation) and PARP(s) activity assay

Culture medium of control and treated cells was substituted with 56 mM HEPES buffer pH7.5, containing 28 mM KCl, 28 mM NaCl, 2 mM $MgCl_2$, 0.01% digitonin, 0.1 mM PMSF and a 1:25 dilution of the cocktail of protease inhibitors, at 5 × 10^6 cells/ml, and incubated with 5 μCi/ml [^{32}P]-NAD^+ (1000 Ci/mmole) alone or in the presence of 5 μM PJ34 at 37 °C for 60 min. Then, cells were scraped off the plates, transferred to eppendorf tubes containing v/v ice-cold 50% TCA and after 3 h standing on ice, collected by centrifugation at 10,600 × *g* for 10 min; finally pellets were washed twice with 5%TCA and three times with ethanol. The cellular pellets were resuspended in 40 mM Tris–HCl pH 7.8, 0.6 mM EDTA, 30 mM $MgCl_2$, 0.05% Triton X-100, 1 mM β-mercaptoethanol, 20% glycerol, 1 mM PMSF and a 1:25 dilution of the cocktail of protease inhibitors and stored at −20 °C until use. [^{32}P]-PAR incorporated in the TCA insoluble fraction was counted in a BECKMAN LS8100 liquid scintillation spectrometer.

Furthermore, [^{32}P]-PAR acceptor proteins were separated by 5–15% SDS-PAGE, and after electroblotting on PDVF membrane, analysed by autoradiography and immunodetection. [^{32}P]-PAR was detached from an aliquot of the same protein sample by incubation at 60 °C for 3 h in Tris–NaOH pH 12, 1 mM EDTA, extracted with $CHCl_3$/isoamyl alcohol (24:1), dried in a SpedVac concentrator, dissolved in 50% urea, 25 mM NaCl, 4 mM EDTA pH 7.5 and subjected to 20% PAGE and autoradiography.

PARP(s) specific activity was determined in a reaction mixture (final volume 50 μl) containing 40 mM Tris–HCl pH 8, 1 mM β-mercaptoethanol, 30 mM $MgCl_2$, 200 μM [^{3}H]NAD^+ (100,000 cpm/nmole) and, as the enzyme source, an aliquot (50 μg of proteins) of cells suspended in 40 mM Tris–HCl pH 7.8, 0.6 mM EDTA, 30 mM $MgCl_2$, 0.05% Triton X-100, 1 mM β-mercaptoethanol, 20% glycerol, 1 mM PMSF and a 1:25 dilution of the cocktail of protease inhibitors, and subjected to mild homogenisation. In reported experiments PJ34 0.01–1 μM was added before the NAD substrate. After 15 min incubation at 30 °C the reaction was stopped by the addition of ice-cold trichloroacetic acid 40% (v/v) and the acid-insoluble material, was recovered on a MILLIPORE filter (HAWP 00010; 0.45 μm) and counted in a BECKMAN LS8100 liquid scintillation spectrometer. One mU is defined as the amount of enzyme catalysing the incorporation of 1 nmole/(min ml) of ADPribose into acid-insoluble material. The data are means of three experiments done in duplicate ±S.E.

2.6. Autoradiographic and immunological analyses

Aliquots of 120 μg of cellular/nuclear proteins were separated by SDS-PAGE (5–15% gradient gels) and either stained with Coomassie or transferred onto a PVDF membrane using an electroblotting apparatus (BIO-RAD).

The membrane was subjected to autoradiographic analysis by the PhosphorImager (BIO-RAD) and/or to immunodetection after blocking with 3% non-fat milk in TBST for 1 h, with antibodies anti-PARP-1 (diluted 1:2000), antibodies anti-PAR (diluted 1:20,000), anti-p53 (diluted 1:2000), anti-AIF (diluted 1:1000), anti-α-actin (diluted 1:1000). As secondary antibodies goat-anti-mouse, or goat-anti-rabbit IgG HRP-conjugate (diluted 1:10,000–1:20,000) in 1% (w/v) non-fat milk in TBST were used. Peroxidase activity was detected using the ECL Advance Western Blotting Kit of GE HEALTHCARE, Italy and quantified using the Immuno-Star Chemiluminescent detection system GS710 (BIO-RAD).

3. Results

3.1. Effects of cDDP +/− PJ34 on the growth of human carcinoma cells

In human carcinoma cell lines of different origin (i.e. HeLa cervical, KB head-neck, HT29 colon) a cDDP dose-dependent

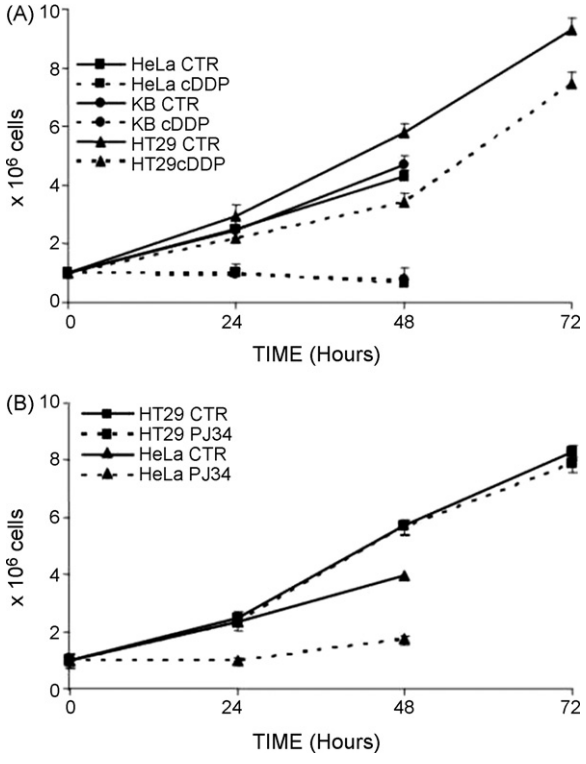

Fig. 1 – Growth inhibition of carcinoma cells subjected to cDDP and PJ34 treatments. (A) Cultures of HeLa, KB and HT29 cells treated with 10 μM cDDP for 4 h and allowed to recover in fresh medium for indicated times. (B) Cultures of HeLa and HT29 cells treated with 5 μM PJ34 for indicated times.

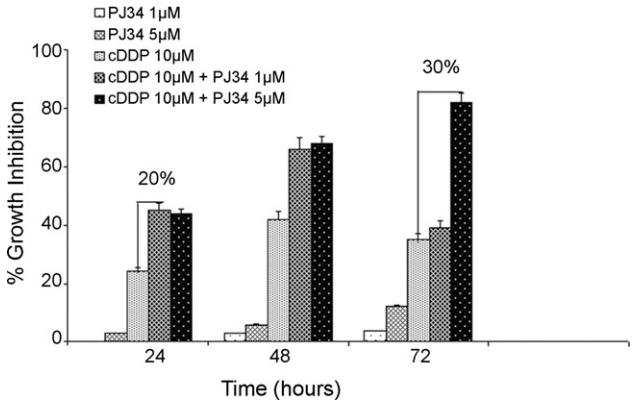

Fig. 2 – Growth inhibition of HT29 cells subjected to cDDP +/− PJ34 combined treatments. MTT assay of HT29 cells treated with10 μM cDDP for 4 h, with PJ34 1–5 μM 24–72 h or with the combinations of both the agents. The synergistic effect is calculated as the difference between the effects of combined treatments and the sum of the two separate treatments; increments are reported as %.

growth inhibition was determined: by dose and time course experiments we selected the condition of 4 h treatment with 10 μM cDDP to compare cell sensitivity to the drug. Fig. 1A shows that 48 h post-treatment induced a 86–88% growth inhibition both in HeLa and KB cells, while 40% of HT29 cells were arrested in their growth following the same treatment and started to recover later on.

Dose-dependent experiments were performed after PJ34 addition to cell culture medium. Fig. 1B shows a comparison of cells sensitivity to 5 μM of the PARPs inhibitor: while HeLa cell growth was drastically inhibited starting from 24 h of culture, HT29 cell growth did not appear to be altered until 72 h.

Thereafter, we evaluated the effect of 4 h treatment with 10 μM cDDP in the presence of 1–5 μM PJ34 on HT29 cell growth. As shown in Fig. 2, by MTT assay we determined a 20% synergistic anti-proliferative effect of 1 μM PJ34, 24 h post-treatment with cDDP; this value increased up to 30% with 5 μM PJ34, 72 h post-treatment.

Moreover, we analysed the degree of cell cycle perturbation in HT29 cells at different times after either single drug or PJ34 and cDDP combined treatments. As shown in Fig. 3, compared to control, cell cycle kinetics was unaffected by 5 μM PJ34 alone, while 10 μM cDDP induced a G_2M cells accumulation at 24 h, that was almost completely reverted at 72 h. Interestingly, 10 μM cDDP + 5 μM PJ34 induced a more sustained G_2M arrest, that was exacerbated at longer times resulting in a drastic alteration of the cell cycle at 72 h.

3.2. *Analysis of PARP-1 activity in cDDP +/− PJ34 treated carcinoma cells*

The involvement of PARP(s) in the signaling of cDDP cytotoxicity was confirmed by monitoring PARP specific activity in control and treated HT29 cells. The enzymatic assay (Fig. 4A) showed, as an effect of a 4 h exposure to 10 μM cDDP, a time dependent activation of PARP, leading to a twofold increase of PARP(s) activity (nmoles of [^{3}H]ADPribose/mg of proteins) 48 h post-treatment. By the same assay we assessed the PJ34 inhibitory efficiency: Fig. 4B shows that the addition to the reaction mixture of 1 μM inhibitor was able to almost completely (<12%) abolish enzymatic activity.

We next identified poly(ADP-ribosyl)ated proteins by labeling permeabilised cells with ^{32}P-NAD (0.01 μM). By autoradiography following separation by SDS-PAGE and electroblotting onto PVDF membrane (Fig. 5A), we found two main bands in correspondence of the MWs of PARP-1 (113 kDa) and core histones (~20 kDa): probing of the same

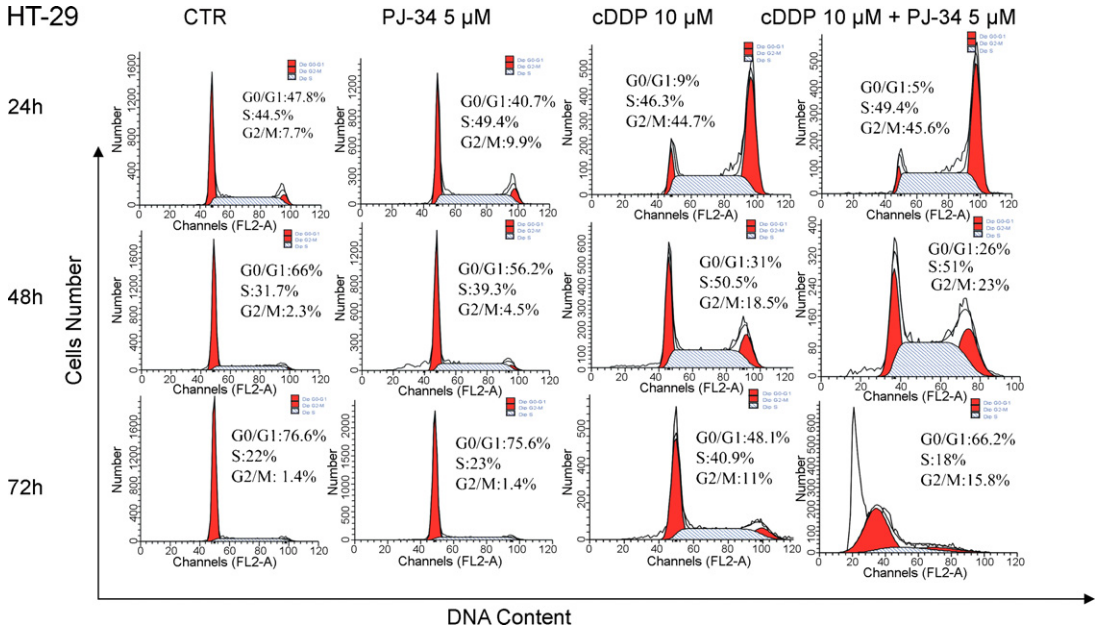

Fig. 3 – Cell cycle progression in HT29 cells subjected to cDDP and PJ34 single and combined treatments. Flow cytometric determination of DNA content after PI staining. The percentage of cells in the different phases of the cell cycle is indicated. Data refer to one of three experiments giving similar results.

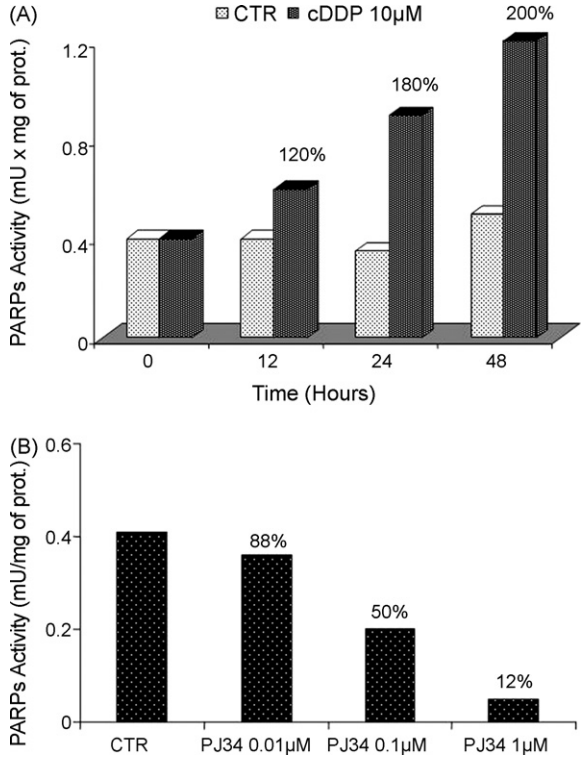

Fig. 4 – PARP(s) activity levels in HT29 cells. (A) PARP(s) activity was measured in homogenates (50 μg of proteins) from control and cDDP treated cells (10 μM 4 h) at different times post-treatment. Data are the means of three different experiments done in duplicate. (B) PARP(s) activity was determined in HT29 cell homogenates (50 μg of proteins) in the presence of different amounts of PJ34 inhibitor. Data are the means of three different experiments done in duplicate.

samples with anti-PARP-1 antibodies confirmed the identity of the main labelled protein as automodified PARP-1. Furthermore, by electrophoretic and autoradiographic analyses of purified ^{32}P-PAR we observed that $(ADP\text{-}ribose)_n$ ranged in size from monomer up to 8–10 ADP-ribose units in chain (data not shown). Interestingly, autoradiographic analysis (Fig. 5A) showed that the extent of PARP-1 modification (number of ^{32}P-PAR polymers/enzyme molecule) changed drastically in treated cells. In fact, although comparable amount of proteins were loaded on the gels (Fig. 5A), the intensities of the autoradiographic bands appeared significantly different in cDDP and cDDP + PJ34 samples, indicating that cDDP stimulates and PJ34 efficiently abrogates PARP-1 activity.

These results were confirmed in intact cells by western blotting analysis of control and cDDP treated HT29 cells with an anti-PAR antibody. Fig. 5B shows a broad immunoreactive band over the MW of PARP-1 identified as a heavily automodified form of PARP-1: long and branched PAR molecules bound to the enzyme induced a drastic electrophoretic mobility shift up to the top of the gel.

Such a band was more evident in the sample from 48 h post-treatment cells than from control cells. In Fig. 5C it is shown that the PAR immunoreactive band was drastically

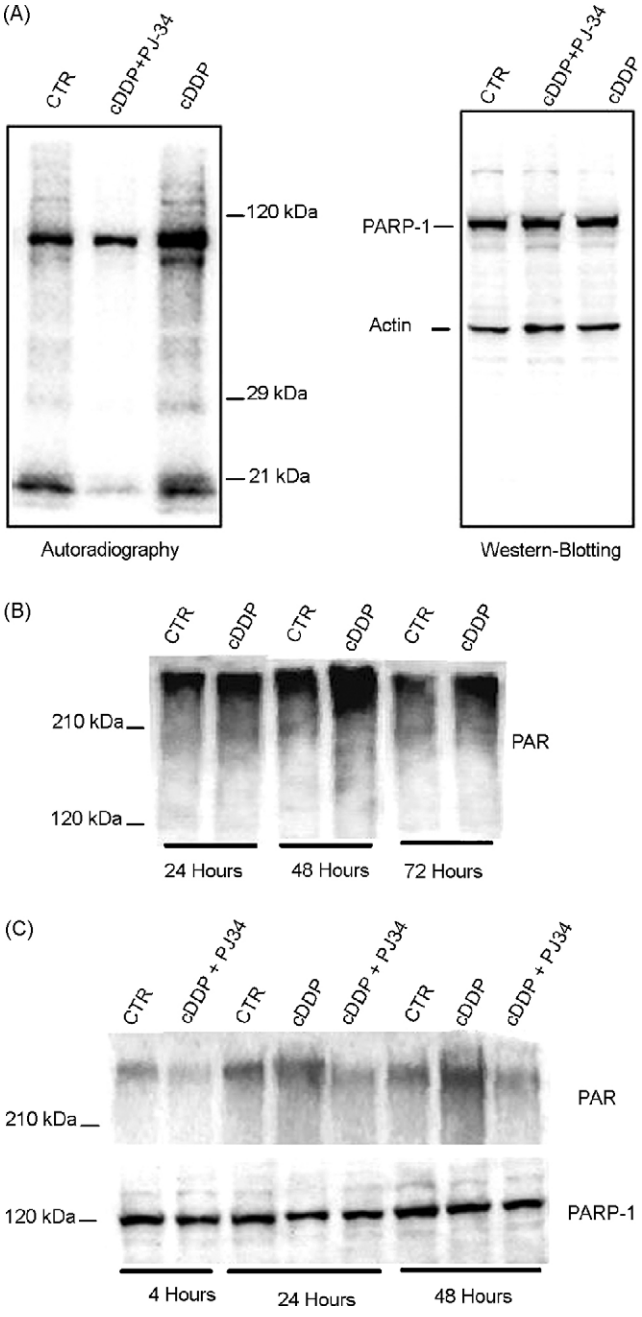

Fig. 5 – PAR acceptor proteins in HT29 cells. (A) Autoradiographic and western blotting analyses of permeabilised HT29 cells incubated with [^{32}P]-NAD$^+$ (0.01 μM). Control cells were compared with those treated for 4 h with 10 μM cDDP +/− 5 μM PJ34. Following 5–15% SDS-PAGE and electroblotting, the PVDF membrane was either subjected to autoradiography or incubated with anti-PARP-1 antibody. Actin served as loading control. (B) Immunodetection of PAR synthesised in cells at different recovery times after 4 h treatment with 10 μM cDDP. (C) Western blotting analysis with PAR and PARP-1 antibodies of homogenates from cells allowed to recover for different times in the presence or absence of 5 μM PJ34, following 4 h treatment with 10 μM cDDP.

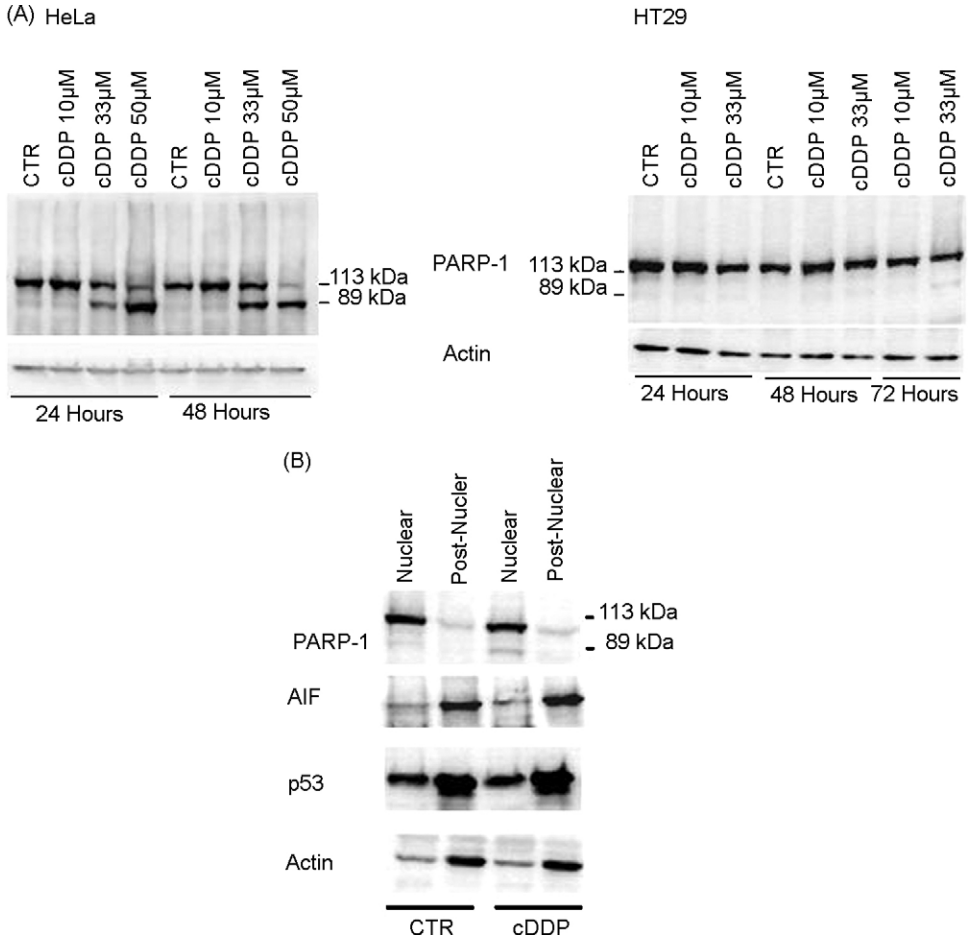

Fig. 6 – cDDP-induced apoptotic pathway in HeLa and HT29 cells. (A) Western blotting analysis of PARP-1 caspase-dependent proteolysis in HeLa and HT29 cells 24–72 h post-treatment with 10–50 μM cDDP. (B) Western blotting analysis of nuclear and post-nuclear fractions from HT29 cells, 72 h post-treatment with 10 μM cDDP, with anti-PARP-1, anti-AIF, anti-p53 and anti-actin antibodies.

reduced after combined treatment with cDDP + PJ34 from 24 to 48 h post-treatment. Furthermore, immunodetection of the same samples with the PARP-1 antibody showed slight differences in the levels of 113 kDa form of the protein, indicating that only a small portion of the enzyme molecules was heavily modified *in vivo*. Western blotting analyses (Fig. 5B and C) show a PAR signal in control samples, that has been considered as the background of such experiments.

3.3. *Apoptotic pathway of cDDP treated carcinoma cells*

By western blot analysis we also evaluated the apoptotic proteolysis of PARP-1 both in HeLa and HT29 cells subjected to higher doses of cDDP. Fig. 6A shows a different apoptotic propensity of the cells, according with their different sensitivity to cDDP: the 89 kDa fragment of PARP-1 was evident in HeLa cells starting from 24 h post-treatment with 33 μM cDDP, while it was hardly visible in HT29 72 h post-treatment with the same cDDP dose.

We than performed the same western blotting analysis after isolation of the nuclear and post-nuclear fraction of such cells and observed (Fig. 6B) the 89 kDa fragment of PARP-1 in the nuclei, showing the onset of an apoptotic death also in HT29 cDDP resistant cells.

On the light of these results we decided to better define such an apoptotic pathway: analysis of several apoptotic markers in nuclear and post-nuclear fractions (Fig. 6B) confirmed the expression of an inactive form of p53, that was mainly cytoplasmic. Moreover, control and treated cells showed a comparable amount of the mitochondrial apoptosis inducing factor (AIF) in the cytoplasm. Thus, the apoptotic pathway involved appears to be p53- and AIF-independent but caspases- and PARP-1 dependent.

4. Discussion

In the present paper we show that the inhibition of PARP activity can be considered a tool even in the challenge to reverse chemo-resistance of carcinoma cells to platinum compounds.

With the aim to explore such a possibility, different human carcinoma cell lines, such as HeLa and HT29, were treated with different doses of cDDP alone and in combination with the

novel PARP inhibitor PJ34. By cell growth inhibition, cell cycle kinetics and the degree of apoptosis these two cell lines exhibited differential sensitivities to the drugs; KB cells that were also analysed, behaved similarly to HeLa cells. In HeLa cells, cytotoxicity induced by cDDP treatment resulted in a dose dependent stimulation of apoptosis, while in HT29 cDDP had a cytostatic effect that appeared to be enhanced by PJ34 at not-cytostatic dose per se. Intriguingly, the two cell lines also showed a different dose-dependent sensitivity to PJ34 alone.

Furthermore, we observed that the p53 mutation of the cells does not correlate with their sensitivity to cDDP. We confirmed that in HeLa cells p53 is not expressed, while in HT29 cells the over-expression of a p53 inactive form correlates with the absence of its target protein p21 (data not shown). Likewise, as already known for HeLa cells, we found in HT29 a cDDP dependent G2/M arrest of the cell cycle, showing that p53 was not necessary for it to occur. In addition, in such cells, we observed that the combination of PJ34 with cDDP determined a more drastic and prolonged arrest of the cells cycle (>72 h), according with the increment observed in cell growth inhibition.

Thereafter, we investigated the involvement of PARP(s) in the signaling of DNA damage induced by cisplatin. It has been recently reported that in xenograft tumor models a potent PARPs inhibitor potentiated the cytotoxicity of cisplatin, carboplatin, and cyclophosphamide, causing regression of established tumors [19]. However, in contrast with the better understood role of PARP inhibitors as adjuvant of alkylating agents [12–14], the mechanisms that underlies their potentiation of cisplatin action still needs investigation [14,18].

In HT29 cells we first got evidences of a stimulation of PAR synthesis and of both PARP-1 auto-modification and histone(s) hetero-modification. In particular, by labeling with ^{32}P-NAD permeabilised cells we identified ^{32}P-PAR acceptor proteins modified by oligo-ADPribose molecules, the main product of a poly(ADP-ribosyl)action reaction at a reduced NAD concentration after cell permeabilisation [20]. Interestingly, both PARP-1 catalysed auto- and hetero-modification reactions appeared to be inhibited by cDDP + PJ34 combined treatments.

Furthermore, by the use of anti-PAR antibodies we observed in intact cells a cDDP-dependent synthesis of long PAR molecules covalently linked to PARP-1, as shown by the electrophoretic mobility shift of the protein. Indeed, at least 50% of PAR synthesised was inhibited by 5 μM PJ34 as long as 48 h after 10 μM cDDP treatment.

By the all of these results, we concluded that the same mechanism of DNA breaks signaling [4] is activated by the cells for the processing of DNA cross-links generated by cisplatin. At present several repair systems are implicated as important for cisplatin-induced DNA damage. Indeed NER is the more important while, at least in ovarian and colon cancer, mismatch repair (MMR) is a small contributor even compared to homologous recombination (HR) [16,21,22]. Recently, the role of PARP in NER was highlighted by a decreased clonogenic survival of UV-irradiated NER-competent cells depleted of PARP [23].

Conversely, it was shown that inhibition of the DNA-PK expression enhanced the sensitivity of cells to cDDP, suggesting that the non-homologous end-joining (NHEJ) system might play a role in cDDP resistance [24]. Indeed, DNA-PK is involved in the PAR dependent DNA damage signaling network: PARP-1−/− cells are shown to be sensitive to the DNA-PK inhibitors and DNA-PKcs or Ku80 defective cells shown to be sensitive to PARP inhibitors [25,26].

Moreover, we observed that cisplatin sensitivity of cells correlates with their apoptotic propensity. In cells treated with increasing doses of cisplatin, on the basis of the appearance of the 89 kDa PARP-1 fragment, we found that an increasing number of cDDP sensitive HeLa cells, against very few of cDDP resistant HT29 cells, entered apoptosis. Furthermore, by analysing isolated nuclei from these latter cells, we confirmed the caspase-dependent proteolysis of PARP-1, showing that cisplatin-induced cell death occurs *via* apoptosis both in sensitive and resistant cells.

As a contribution to the still pending question, whether cisplatin-induced cell death is always produced by apoptosis [21], we further analysed the apoptotic behaviour of cDDP-treated cells. We got evidences of a pathway: (i) p53-independent, as demonstrated by the fact p53 is mutated and cytoplasmic in the cells under study; (ii) AIF-independent, on the basis of the cytoplasmic localisation of this mitochondrial factor. Recently, it has been shown that AIF translocation into the nucleus can mediate both a caspase-dependent and independent cell death [27,28]. Our evidence of the PARP-1 fragmentation by caspases as a result of cDDP cytotoxicity, agrees with the induction of a caspase-dependent, mitochondrial-independent, apoptotic pathway.

In conclusion our findings demonstrated that the DNA damage signal arising from cDDP action is gathered by PARP-1, suggesting that a combination of PARP inhibitors and cisplatin could be effective in ameliorating the effects of the chemotherapic agent on carcinoma cells, according to a strategy based on the restoration of apoptosis in neoplastic cells, independently of their p53 status.

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