Mitotane sensitizes adrenocortical cancer cells to ionizing radiations by involvement of the cyclin B1/CDK complex in G_2 arrest and mismatch repair enzymes modulation

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Abstract. Mitotane inhibits steroid synthesis by an action on steroidogenic enzymes, as 11ß-hydroxylase and cholesterol side chain cleavage. It also has a cytotoxic effect on the adrenocortical cells and represents a primary drug used in the adrenocortical carcinoma (ACC). H295R and SW13 cell lines were treated with mitotane 10^{-5} M and ionizing radiations (IR) in combination therapy, inducing an irreversible inhibition of cell growth in both adrenocortical cancer cells. As shown in a previous report, mitotane/IR combination treatment induced a cell accumulation in the G_2 phase. Here, we report the radiosensitizing properties of mitotane in two different ACC cell lines. The drug reveals the effectiveness to enhance the cytotoxic effects of IR by attenuating DNA repair and interfering on the activation of mitosis promoting factor (MPF), mainly regulated by the degradation of cyclin B1 in the mitotic process. These events may explain the inappropriate activation of cdc2, implicated in G_2/M phase arrest and probably induced by the mitotane and IR in the combined treatment. Indeed, treatment with purvalanol, a cdc2-inhibitor prevents cell cycle arrest, triggering the G_2/M transition. The observation that mitotane and IR in combination treatment amplifies the activation level of cyclin B/cdc2 complexes contributing to cell cycle arrest, suggests that the MPF could function as a master signal for controlling the temporal order of different mitotic events. Moreover, we report that mitotane interferes in modulation of mismatch repair (MMR) enzymes, revealing radiosensitizing drug ability.

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Introduction

Sporadic adrenocortical carcinoma (ACC) is an uncommon tumour which rarely occurs with synchronous bilateral adrenal involvement (1). In advanced disease, highly individualized treatment include surgical mass reduction, control of endocrine activity, and alleviation of symptoms from local tumour growth (2-4).

Mitotane, 1,1-dichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane (o,p'-DDD), is a compound which represents the effective agent which blocks cortisol synthesis by inhibiting 11ß-hydroxylation and cholesterol chain cleavage thus representing an effective agent in the treatment of the functional ACC (4). The drug acts selectively on the adrenal cortex producing a specific cytotoxic through free radical production (5). Moreover, it supports several effects connected to many biological processes such as energetic metabolism, stress response, cellular structure and tumorigenesis in H295R human adrenocortical carcinoma cell line (6). In clinical employment, it is usually well tolerated in the plasmatic narrow range between 14 and 20 mg/l. Mitotane produces a dose-related cellular toxic effect on adrenocortical cells, by producing focal degeneration, particularly in the fascicular and reticular zone, and relatively in the glomerulosa zone, causing mitochondrial damage. The mitotane use is limited by its adrenotoxic effect and that some of the treated patients shows side effects, particularly gastrointestinal and neurological ones (4).

We recently observed that the combination of ionizing radiations (IR) plus mitotane induced an inhibition of cell growth with a cell accumulation in the G_2/M phase of cell cycle accompanied by an increase of cyclin B1 protein and high kinase activity (7).

We proposed mitotane as an effective ionizing radiation sensitizer *in vitro*: its radiosensitizing properties induce molecular modifications such as proteins, metabolites, changes

in cell cycle distribution and cell cycle arrest. In fact the ACC cells only treated with IR overcome this treatment, recycling normally, whereas the cells only treated with mitotane compound did not show substantial modifications in cell cycle progression.

Several studies consider the ubiquitin-proteasome pathway as the major system for selective degradation of short-lived regulatory proteins in eukaryotic cells (8,9). This pathway modulates the levels of target proteins and/or compositions of multi-protein complexes in cells by targeted protein degradation.

Cyclin B1 is the regulatory subunit of the Cdk1 kinase (named also Cdc2) required for mitotic initiation (10). During cell cycle progression, the levels of cyclin B1 are mainly regulated at the transcription and proteolysis levels. Cyclin B1 accumulation begins in S phase and continues throughout G_2 phase until mitosis. By contrast, cyclin B1 transcription is repressed for blocking mitotic entry during the G_2 checkpoint induced by DNA damage (11-13). Thus the cyclin B1 protein must be degraded in order to permit G_2/M transition and its proteolysis is essential for Cdk1 activity to end (14). Recently, it was reported that the mitosis trigger is mainly due to the removal of Cdk1 inhibition, and assessed that one-third of endogenous MPF was sufficient to inhibit cell cycle progression (15-17). In this study we used a specific pharmacological inhibitor of cdk: purvalanol-A; it is a protein kinase inhibitor with high selectivity for Cdk2 and Cdk1 (18), acting through competitive inhibition of ATP binding (19). We provide evidence that the combination IR-mitotane induces a block of G_2/M phase in cell cycle, due to reduction of DNA repair and above all to an excessive MPF activity implied in the correct cell cycle progression, in temporal order of mitotic events.

MMR is a highly conserved repair system that corrects mismatches arising during DNA replication and safeguards genomic integrity (20,21). MMR consists of at least seven proteins, including hMLH1, hMLH3, hPMS1, hPMS2, hMSH2, hMSH3 and hMSH6. Studies over the last decade have suggested that MMR function is also implicated in other cellular processes, such as transcription-coupled repair and damage responses to chemical and physical agents (22,23).

Therefore, we reveal the evidence that mitotane inhibits the MLH1 and MSH2 MMR protein levels, suggesting strong properties of this drug in radiosensivity of ACC.

Materials and methods

Cell culture and treatment. H295R and SW13 cell lines were supplied from the American Type Culture Collection (ATCC, Rockville, MD, USA). Dimethylsulfoxide, in cell culture medium, was adjusted to 0.03% at the final concentration. H295R steroid synthesizing cells were cultured in DMEM/HAM'S F-12, medium supplemented with penicillin/streptomycin 50 U/ml, enriched with a mixture of insulin/transferring/selenium and 10% NuSerum-I. SW13 cells were grown in Leibovitz's L-15 medium supplemented with 10% bovine serum. Cell lines were irradiated by a Varian Clinac 600c/d 6MV photon beam. Scanditronix FC65G farmer ionisation chamber was used to evaluate the beam properties in water and in polymethylmethacrylate (PMMA) phantom. The cell irradiation was based on single irradiation doses of 6 Gy/min and analyzed from 24 to 120 h. All experiments were repeated at least three times and each experimental sample was seeded in triplicate. Radiation treatment was given 24 h post-seeding and then the cells were treated with mitotane 10 μM (Sigma-Aldrich). The viable cells were counted using a hemocytometer by trypan blue exclusion. The cell treatment with purvalanol-A were carried out by adding 10 and 20 μM to cells after ionizing radiation and mitotane treatment.

Cell cycle analysis. Cell cycle was studied by using both bromodeoxyuridine incorporation (BrdU; Sigma Chemical 90 Co., St. Louis, MO) and propidium iodide (PI) staining. Both BrdU pulse-labelling and continuous-labelling experiments were carried out. Pulse-labelling experiments were performed by adding 10 μl BrdU to the medium during the last 30 min before analysis. For BrdUrd continuous-labelling experiments, cells were continuously exposed for 50 h before analysis. After 30 min and after 50 h, cells were harvested, washed once in PBS, fixed in 70% ethanol and stored at 4˚C before analysis. Samples were then incubated with mouse monoclonal antibody anti-BrdU (Roche Diagnostics, Milan, Italy) in complete medium containing 20% FCS and 0.06% Tween-20 (Calbiochem, San Diego, CA) at room temperature for 1 h. After washing in PBS, cells were incubated with FITC-conjugated rabbit anti-mouse IgG (Dako, Glostrup, Denmark) in PBS for 1 h. Finally, cells were stained with a solution containing 5 μg/ml PI and 75 KU/ml RNase in PBS for 3 h. The top line of the cytograms represent BrdUrd-positive cells. In order to perform PI staining, treated and untreated cells were fixed in 70% ethanol and stained with a solution containing 50 μg/ml PI (Sigma Chemical) and 75 KU/ml RNase (Sigma Chemical) in PBS for 30 min at room temperature. For both experiments twenty thousand events per sample were acquired by using a FACScan cytofluorimeter (Becton-Dickinson, Sunnyvale, CA, USA).

Western blotting. Cellular lysates were sonicated on ice, clarified by centrifugation at 20,000 x g and stored at -80˚C. An aliquot of the cell lysates was used to evaluate the protein content by colorimetric assay. Total protein content (70 μg) was electrophoresed on 10% polyacrylamide gel in the presence of SDS and transferred onto a nitrocellulose membrane. Blots were blocked for 1 h at room temperature with 5% non-fat dry milk in T-PBS buffer. Treated and untreated cells were incubated with the anti-MLH1, MSH2 (Santa Cruz Biotechnology, CA, USA) and phospho-histone1 (Cell Signalling Technology, Inc., MA, cat 06-597). The visualization of the antigens was done by enhanced chemiluminescent detection reagents by ECL.

Co-immunoprecipitation from cultured ACC H295R and SW13. Cell pellets were resuspended in cell lysis solution at a low stringency (NP40 1%, 103, leupeptin 1 μg/ml, pepstatin 1 μg/ml, aprotinin 2 μg/ml, phenylmethylsulfonylfluoride (PMSF) 0.2 mM, sodium fluoride 10 mM) and a protease inhibitor. Then, samples were sonicated for 10 sec (Branson sonifier 150). Preclearing of the lysates was done by adding protein A to the extracts and mixing for 1 h at 4˚C. After preclearing, supernatant was again incubated with the protein A

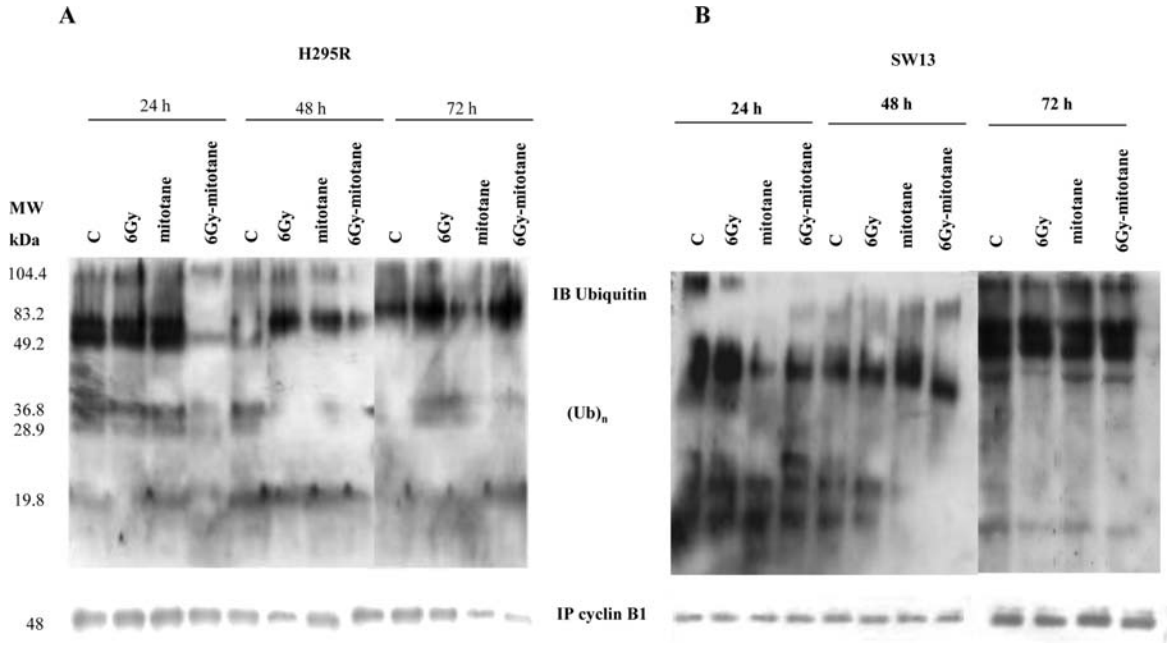

Figure 1. Degradation of cyclin B1 after treatments. H295R cell lysates (A) and SW13 cell lysates (B) were immunoprecipitated (IP) with cyclin B1 at 24, 48 and 72 h after the different treatments and precipitated proteins were immunoblotted with anti-ubiquitin antibody α-Ub.

and with cyclin B1 (BD Biosciences USA cat 554178) at 4˚C overnight. Ubiquitin protein (Santa Cruz Biotechnology) was detected by Western blot analysis using chemiluminescence with a rabbit polyclonal antibody.

Apoptotic assay. H295R and SW13 cells were cultured in chambers at a density of $0.015x10^6$ and $0.04x10^6$ respectively in a total volume of 0.2 ml. Cells were treated with and without 10-20 μM purvalanol-A, at 24 and 48 h, DNA strand breaks were labeled by TUNEL assay (*In Situ* Cell Death Detection Kit, Roche), and cellular apoptosis was analysed by light microscopy.

Statistical analysis. The data are presented as means ± SD. A comparison of the individual treatment was conducted by using Student's t-test. A $p<0.05$ was considered significant.

Results

Cyclin B1 is not involved in cell cycle arrest. Considering that the protein ubiquitination regulates many cellular processes, including protein degradation, signal transduction, DNA repair and cell division, we sought to exclude the possibility that mitotic arrest was caused by overexpression of cyclin B1 protein, the cells were tested for cyclin B1 ubiquitination after different treatments for both H295R and SW13 cell lines. An immunoblot analysis of anti-cyclin B1 immunoprecipitates was performed as shown in Fig. 1. The results revealed that after different treatments from 24 until 72 h cyclin B1 was polyubiquitinated. Therefore, we concluded that the majority of endogenous cyclin B1 was degradaded, as some authors reported, and a small amount of active cyclin B1 is sufficient to inhibit the M phase transition, indicating that the activity of the cyclin B1 and its catalytic subunit cdk-1 was necessary for triggering the mitotic event (14).

Effects of purvalanol-A in growing cells. H295R and SW13 cell lines were exposed to purvalanol-A at different concentrations 10 and 20 μM, to evaluate its effects on cell growth at different times (24, 48 and 72 h).

Cells were harvested and counted by using the trypan blue dye exclusion test, to evaluate the cell viability. As shown in Fig. 2A, both purvalanol-A concentrations had an anti-proliferative effect. In fact purvalanol-A 10 and 20 μM reached 48 and 76% of inhibition respectively at 72 h in H295R cells. In SW13 cell line we observed 81 and 85% of inhibition at 10 and 20 μM, respectively, after 72 h from treatment, exerting minimal effect on cell viability in the cell lines (data not shown). In order to examine if purvalanol-A concentrations 10 and 20 μM affected the cdk-1 activity, the kinase activity was evaluated. Since histone H1 is known to be a cdk-1 substrate for phosphorylation, measurements of its phosphorylation level were evaluated (15).

In the Western blots obtained from cells exposed to different concentrations of purvalanol-A, we observed in H295R cell line a reduction of H1-phosphorylation expression of 3-fold ($p<0.05$) in cells treated with 20 μM of purvalanol-A and of 1.5-fold ($p<0.05$) in cells treated with purvalanol-A 10 μM already at 4 h of treatment. After this time there was a small increase in protein expression as shown in Fig. 2C, probably connected to cellular recovery, as shown in Fig. 2A at 72 h. Since the 20 μM purvalanol-A concentration led to a stronger inhibition of cdk-1 activity than 10 μM concentration, we also verified if this concentration was efficient to inhibit cdk-1 activity. As shown in Fig. 2C purvalanol-A at 20 μM blocks cdk-1 activity until 72 h.

In SW13, 2.5-fold ($p<0.05$) and 1-fold ($p<0.05$) decrease in H1 phosphorylation was observed in cells treated with purvalanol-A 20 and 10 μM respectively at 4 h (Fig. 2D). After 24 h from treatment a decrease of pH1 expression level was observed in respect to control until 72 h (Fig. 2D).

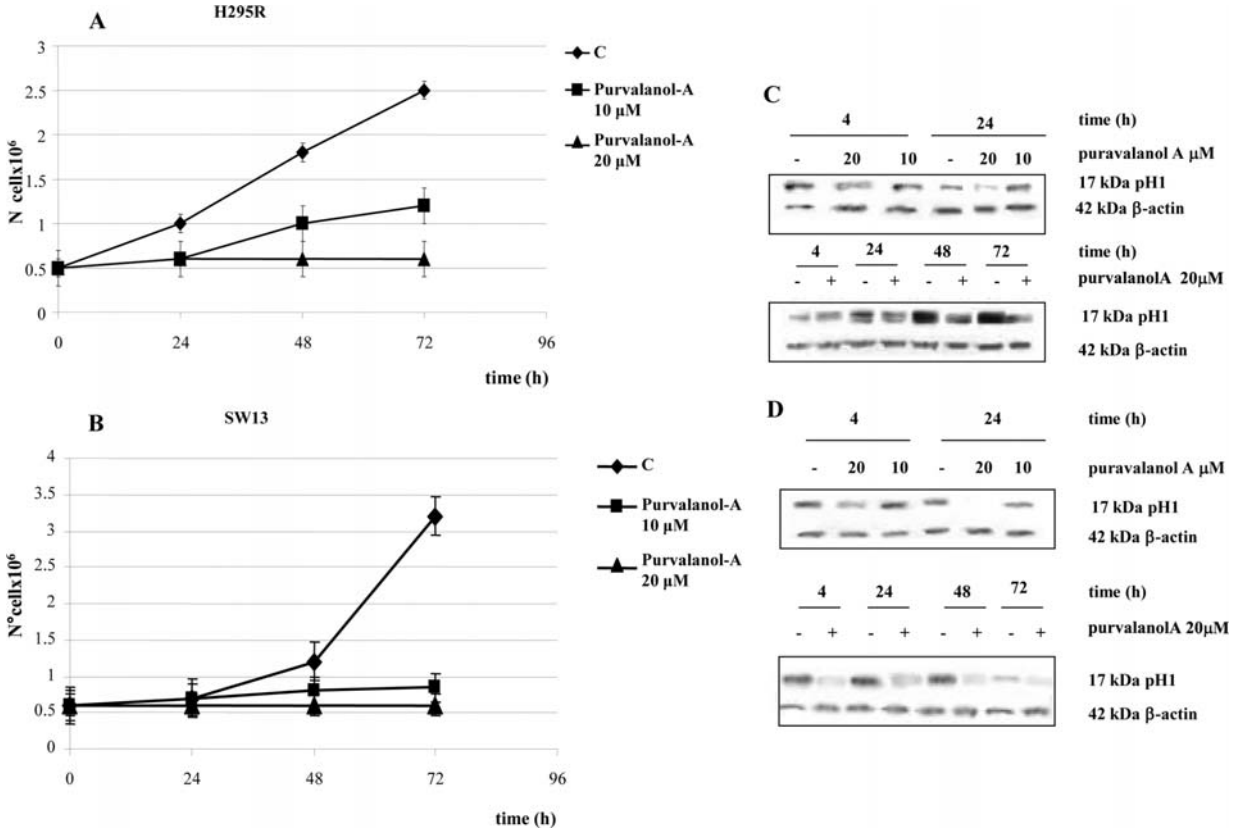

Figure 2. Effect of purvalanol-A on H295R (A) and SW13 (B) cell line proliferation. Both cell lines were treated with two different concentrations of purvalanol-A: 10 and 20 μM for 72 h and the adherent cells were counted. Purvalanol-A in H295R and SW13 treated cells induced growth inhibition. Effect of different purvalanol-A concentrations on CDC2 kinase activity of H295R (C) and SW13 (D) cell lines after different time of treatment. The Western blot analysis of the expression of phospho-H1 was performed by histone H1 phosphorylation. ß-actin was used as control for loading equal protein amounts.

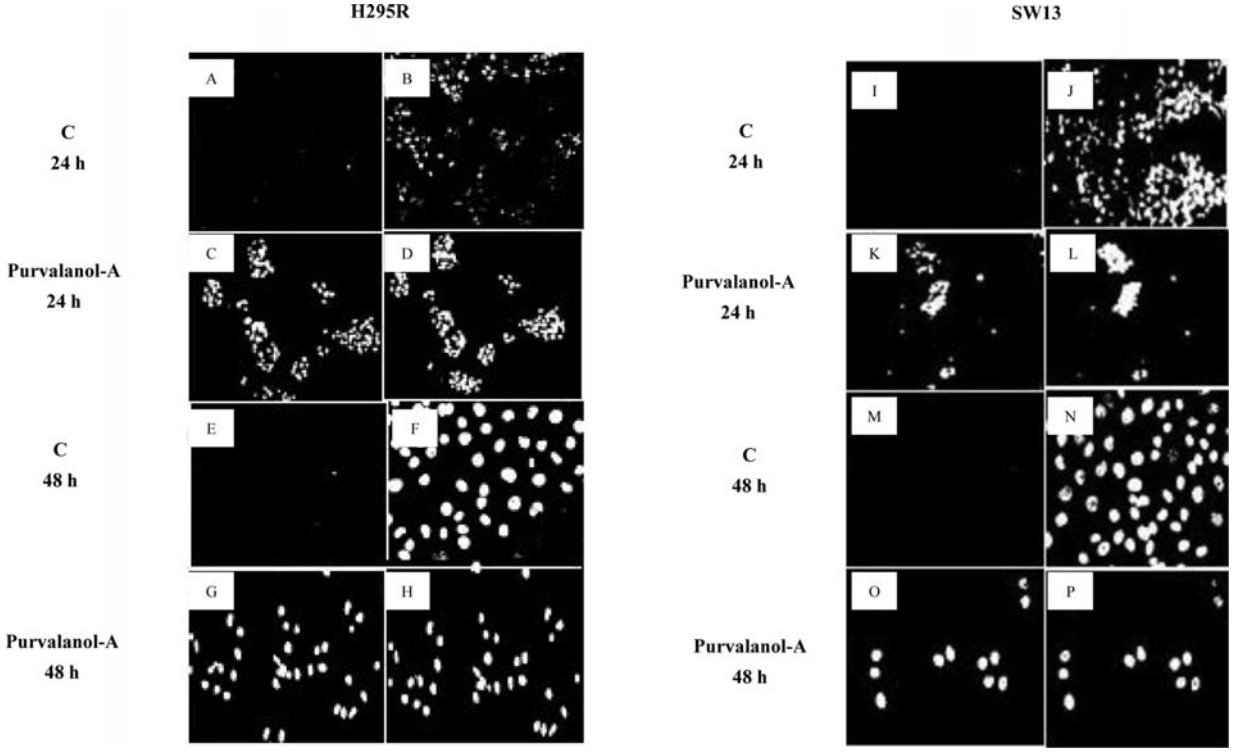

Figure 3. Fluorescence microscopy photographs of nuclei of treated and untreated cells with 20 μM purvalanol-A for 48 h. H295R and SW13 cell lines were analysed for apoptotic cells by TUNEL assay (left panels: A, C, E and G and I, K, M and O, respectively). Hoechst stains was used for nuclear DNA (right panels: B, D, F and H and J, L, N and P). Fragmented nuclei emitting intense fluorescent staining were considered apoptotic.

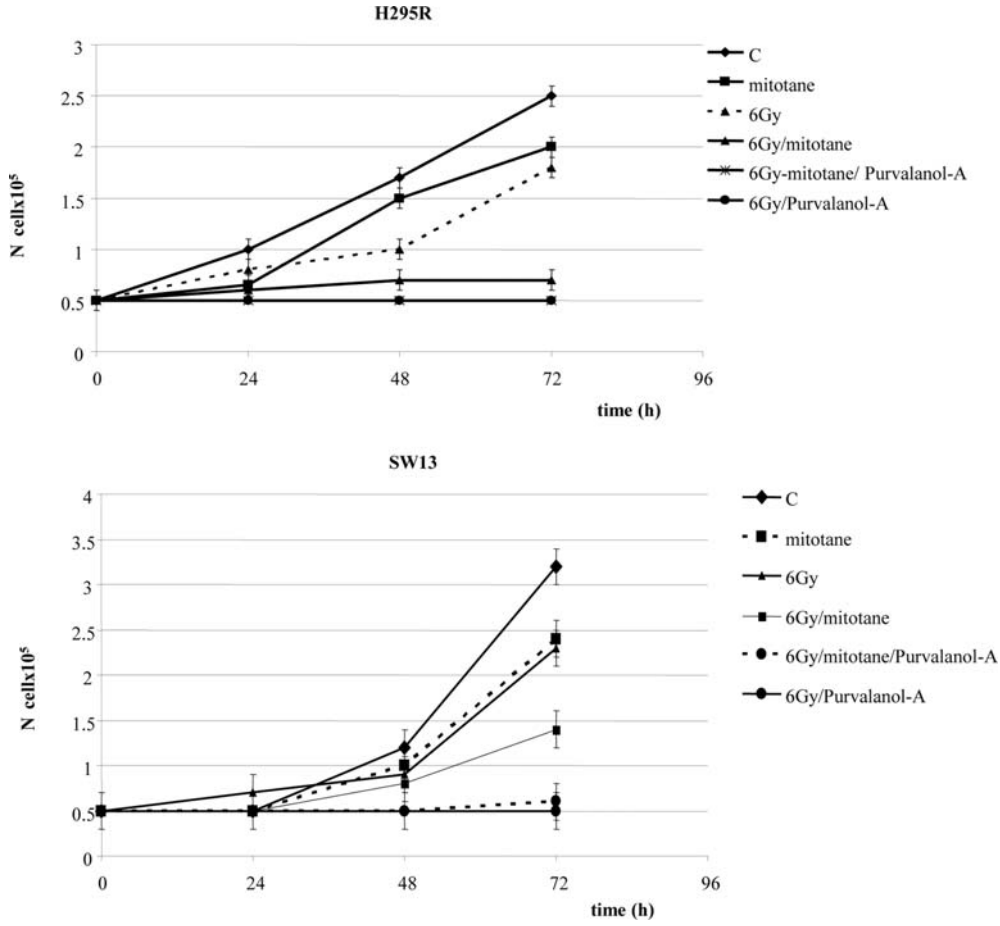

Figure 4. Effect of purvalanol-A in combination of mitotane and ionizing radiation (IR) treatment on adrenocortical cancer cell proliferation. The cdc2-kinase inhibitor in combined treatment with mitotane and IR enhanced cell growth inhibition either in H295R (A) or SW13 (B).

Since the cdk-1 activity was inhibited until 72 h with purvalanol-A at 20 μM, this concentration was used in our experiments. Therefore, TUNEL assay showed by fluorescence that 20 μM of purvalanol-A induced apoptosis on both adrenal cancer cell lines from 24 until 72 h as shown in Fig. 3C, G, K and O. This in accordance with some authors, who reported that purvalanol-A interfered in the synthesis of antiapoptotic proteins (17).

As reported in our previous work (7) we have reconfirmed that in H295R the mitotane treatment did not induce relevant changes in cell growth. When the cells were treated with the 6 Gy there was an inhibition of 28%, whereas the 6 Gy-mitotane in combination induced 80% of inhibition at 72 h. In SW13, we also observed that mitotane treatment did not induce a marked effect on cell growth, instead the 6 Gy and 6 Gy-mitotane treatments induced an inhibition of 24 and 58%, respectively. Moreover, we have treated exponentially growing H295R and SW13 cells exposed to 6 Gy and 6 Gy-mitotane plus purvalanol-A (Fig. 4). The effects of these treatments suggested that purvalanol-A inhibits cdk-1 activity and induced cell growth inhibition triggering apoptosis.

Purvalanol-A enhances cell death by inhibiting up-regulation of cdc2 kinase activity after 6 Gy-mitotane-treated cells. In a recent work we previously found that 6 Gy-mitotane combined therapy induced G_2/M cell arrest associated with the formation and activation of the cyclin B1/cdk1 complex (elevated levels of cyclin B1 and high kinase activity) on both H295R and SW13 cell lines (7). Since the G_2/M arrest is classically associated with decreased levels of cyclin B1 and the inactivation of the cyclin B1/cdk1 complex, the obtained result suggests that the G_2/M arrest in both H295R and SW13 cells did not depend on low cyclin B1 levels. On the other hand it is well known that the entry into mitosis requires both the formation and activation of the cyclin B1/cdk1 complex, therefore we examined if the 6 Gy-mitotane combination induced G_2/M arrest could be due to a different biological event able to maintain cyclin B1/cdk1 complex in an activated form. Consequently, we tested if purvalanol-A, a cdk1 inhibitor, could rescue the 6 Gy-mitotane G_2/M arrest. In 6 Gy-mitotane treated cells, without purvalanol-A administration, we observed a marked G_2/M arrest. Six Gy-mitotane treatment induced an accumulation in the G_2 phase of cell cycle in both cell lines (50 and 30% in H295R and SW13, respectively), compared with controls (17 and 7%, respectively) already after 24 h. This G_2 arrest was irreversible since, even 72 h after treatment, the G_2 accumulation was still evident (67 and 27% in H295R and SW13 cell lines, respectively), indicating that the 6 Gy-mitotane treated cells were not able to recover from the 6 Gy induced G_2 arrest. Moreover, in 6 Gy-mitotane combined treatment any presence of the sub-G_1 fraction,

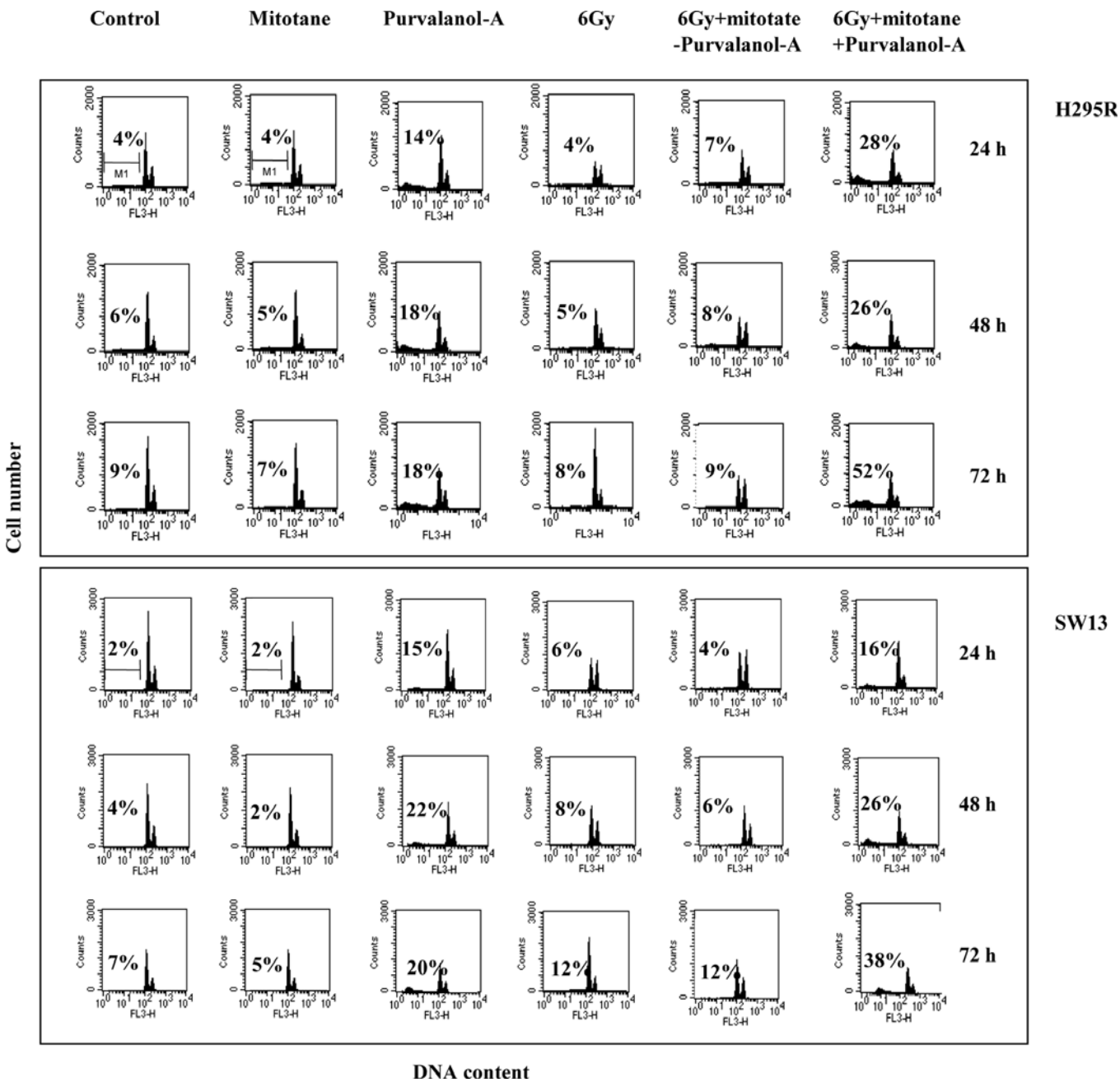

Figure 5. Cell cycle perturbation of untreated and treated H295R and SW13 cell lines by using FCM. Both cell lines were treated with mitotane and/or purvalanol-A, given alone or in combination and the analysis was performed at the indicated time. DNA content is represented on the x-axis and the number of cells counted is reported on the y-axis. The sub-G_1 peak (marker M1) represents the percentage of apoptosis. Twenty thousand events were acquired for each sample. The data are representative of two separate independent experiments with similar results.

which is typical of apoptotic cell death, occurred. On the contrary, purvalanol-A in 6 Gy-mitotane treated cells, resulted in a decrease in the G_2/M fraction consistent with the presence of the sub-G_1 fraction. Apoptosis in purvalanol-A + 6 Gy-mitotane treated H295R cells was more consistent than in SW13 cell line (52 and 38% respectively, at 72 h) indicating that the enhancement of purvalanol-A-induced apoptosis in 6 Gy-mitotane H295R cells could be due to a more consistent G_2/M block than in SW13 cells. In purvalanol-A-treated cells, we evidenced the presence of a sub-G_1 peak (14 and 15% respectively, in H295R and SW13 cells), already at 24 h. The cell fraction in the sub-G_1 peak reached about 18% in both cell lines concomitant with the depletion of the cells from G_1 compartment (Fig. 5). This data suggest that purvalanol-A overcome G_2/M block is due to 6 Gy-mitotane treatment enhancing apoptosis.

hMLH1 and hMSH2 down-regulation increase adrenocortical cancer sensitivity to ionizing radiations. To address whether the ionizing radiation interferes with the repair of IR-induced DNA damage, we have studied MSH2 and MLH1 MMR protein levels after different treatments.

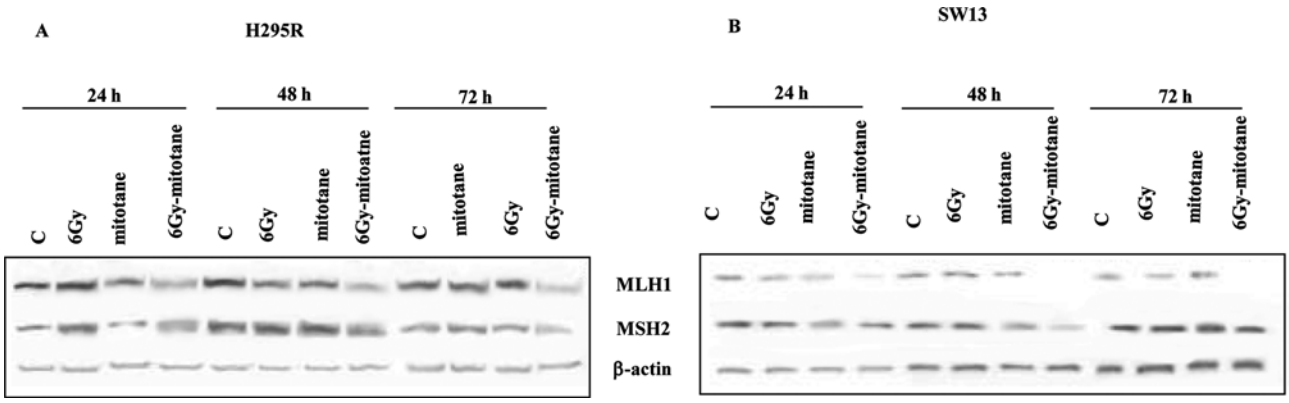

Figure 6. Western blot analysis of MSH2 and MLH1 proteins of H295R cell line (A) and SW13 cell line (B) were evaluated at 24, 48 and 72 h after treatments. Each lane was loaded with 70 μg proteins from lysates and ß-actin was used as control for loading equal protein amounts. The experiment was repeated twice showing similar results. Band intensities were measured by densitometry analysis.

Western blotting of both MLH1 and MSH2 proteins normalized versus ß-actin levels revealed that, in H295R and SW13 cell lines, the exposure of growing cancer cell lines to mitotane treatment, produced a transient down-regulation in the first 24 h of both MSH2 and MLH1. This effect was not maintained during the treatment, in fact the protein levels after this time were comparable with control cells (Fig. 6). While the amount of both proteins showed a significant decrease in the expression levels after 6 Gy-mitotane treatment, the inhibition remained constant during 72 h of treatment at about 2-fold ($p<0.05$) versus control cells. Non-significant alteration was observed in protein expression in 6 Gy treatment on both cell lines to control cells.

These data suggest that mitotane administration interferes with the repair process, sensitising adrenocortical cancer cells to the radiation treatment by inhibiting MLH1 and MSH2 proteins, thus rendering these cells more susceptible to radiation-induced cell cycle arrest in G_2 phase.

Discussion

ACC is a rare cancer with a very poor prognosis. The therapeutic strategies include surgery, considered the mainstay of treatment, which leads to complete healing in early stages of ACC (23). Medical treatment has not substantially changed in the last decades and it still mainly relies on the use of mitotane. It provides a clinical response in one-third of patients and an hormonal reduction in 75% of cases (24). Terzolo *et al* (25) have demonstrated, in a retrospective analysis involving 177 patients with ACC, that adjuvant mitotane may prolong recurrence-free survival in patients with radically resected ACC. At present its side effects are avoidable through the plasmatic dosage of the drug and the possibility of identifying a therapeutic range. Also in the later stages of ACC different therapeutic protocols have been proposed, based on further chemotherapeutic agents in addition to mitotane (26).

Several reports describe a beneficial response exceeding 42% obtained with radiotherapy in ACC treatment (27-29). Although these studies do not comply with current treatment protocols and despite the number of cases being rather small, they clearly show that ACC is not resistant to IR. Patients affected by ACC stage III, treated after adrenalectomy with IR at a dose of 45-55 Gy for 5 weeks, showed a better reduction of recurrence of disease than a group of patients, who had not received radiotherapy (30).

In a previous study (7) we observed a G_2 delay of cell cycle in H295R and SW13 adrenocortical cancer cells after IR and mitotane exposure in combination. This result was accompanied by a cdc2 high kinase activity and an up-regulation of cyclin B1 in all the experiment. Several authors report that the mitosis promoting factor (MPF) inactivation can independently trigger different mitotic events (16). The G_2 checkpoint is a critical cellular defence system that prevents mitotic entry in response to DNA damage. Several mechanisms have been implicated in G_2 arrest such as the level of cyclin B1-Cdk1 activity blockage (11-14).

Conversely, the new synthesis and the cyclin B1 accumulation during S and G_2 phases are required for the full activation of Cdk1 driving into mitosis, whereas cyclin B1 must be destroyed during normal cell cycle progression (10,15).

Xu and Chang (16) believe that the MPF works as a master signal for controlling the temporal order of different mitotic events and its inactivation is necessary for mitosis regulation. In fact the authors reported that cyclin B1 overexpression was responsible for G_2/M block and Stemmann *et al* (31) also found that, in HeLa cells, when the amount of non-degradable cyclin B added to the *Xenopus* extract was increased, sister chromatid separation was blocked (31), assessing that less than 30% of endogenous cyclin B1 level was sufficient to block the mitosis phase. These observations led us to hypothesize that the mitotic arrest was caused by cyclin B1 overexpression. As a consequence we examined the cyclin B1 degradation by focusing our attention on protein ubiquitination.

In this study we observed a polyubiquitination of cyclin B1 in both treated and untreated adrecortical cancer cell lines in all experimental time points. This prompted us to speculate that a smaller amount of cyclin B1 protein is sufficient to maintain enough MPF activity to block the G_2/M transition, suggesting that cyclin B1 stability is a key regulatory factor in initiating G_2 and M arrests in response to cellular damage. In accordance with Xu and Chang (16), these findings led us to hypothesize that our data may represent the threshold

model examined by them. Therefore, it is possible that, slight MPF activity is sufficient to maintain the block of the mitosis entry. In order to confirm the role of the MPF complex in the G/M transition, we evaluated if the mitotic arrest was rescued by applying cdk-1 inhibitors. The inhibitory effect of purvalanol-A against the activity of 22 human kinases in a cell-free system has been tested and it was found that the cdk-1 kinase activity was strongly inhibited at low concentration, thus indicating its high selectivity (17-19). We confirmed this characteristic of purvalanol-A in H295R and SW13 cell lines by observing that the treatment with 20 μM purvalanol-A completely inhibited cdk-1 activity. We also found that the purvalanol-A alone induced cell death by triggering apoptosis and in combined treatment with IR-mitotane, it enhanced the apoptosis induction, by suggesting that this compound could initiate the signal trasduction pathways for apoptosis and could be a clinical target therapy against ACC.

In many types of cancer it has been observed that adjuvant radiotherapy is associated to a long-term survival (32,33). All eukaryotic cells show cell cycle delay after exposure to DNA damaging agents (34,35). IR antiproliferative effects were studied in some cell lines. Ovary and breast carcinoma cells and pituitary adenoma human cell lines have been described to respond to DNA damaging through cell cycle arrest by inducing DNA repair pathways, or through an apoptotic process by activating effector molecules of death (36-38). Nevertheless, few data are available concerning radiotherapy in ACC. Some of them are represented by clinical reports, indicating that radiotherapy is of great benefit to ACC. In a recent study Fassnacht *et al* (33) affirmed that radiotherapy might play a role as an adjuvant therapy after surgery in patients with high risk of local recurrence.

Therefore, we examined molecular consequences of DNA repair factors MMR (mishmatch repair protein) such as MLH1 and MSH2 after exposure to IR, IR-mitotane and mitotane alone, in both H295R and SW13 cell lines. We found that either mitotane or IR-mitotane treatments caused an MMR modulation. In mitotane treated cells, the modulation decreased in the time after treatment and the protein levels were comparable to control cells. Instead in the combined treatment the down-regulation of MLH1 and MSH2 proteins was present in all experimental times. Thus we can postulate that mitotane compound sensitises both H295R and SW13 cell lines to IR.

As previously mentioned, mitotane actually represents the main adrenolytic compound employed in the treatment of patients affected by ACC (4,24).

In conclusion, in our study we observed that IR alone was not powerful enough to induce an antineoplastic effect in H295R and SW13 cell lines. Their sensitivity to radiation was mitotane-dependent and induced an enhancement of the tumour cell response to radiotherapy, by causing a cell cycle delay in G_2/M phase, characterized by high cdc2 kinase activity.

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