Mitotane sensitizes adrenocortical cancer cells to ionizing radiations by involvement of the cyclin B1/CDK complex in G₂ arrest and mismatch repair enzymes modulation

LIDIA CERQUETTI1,2, CAMILLA SAMPAOLI1,2, DONATELLA AMENDOLA2, BARBARA BUCCI2, SILVIA MISITI1,2, GIORGIO RAZA3, UGO DE PAULA1, RODOLFO MARCHESE2, ERCOLE BRUNETTI2, VINCENZO TOSCANO3 and ANTONIO STIGLIANO1,2

1Endocrinology Sant’Andrea Hospital, II Faculty of Medicine, ‘Sapienza’ University of Rome, Via di Grottarossa 1035, Rome 00189; 2Research Center, 3Radiation Oncology Unit, S. Pietro Hospital, Via Cassia 600, Rome 00189, Italy

Received March 16, 2010; Accepted May 11, 2010

DOI: 10.3892/ijo_00000698

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 G₂ 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 G₂/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 G₂/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.

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 G₂/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 Cdce2) 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 G2 phase until mitosis. By contrast, cyclin B1 transcription is repressed for blocking mitotic entry during the G2 checkpoint induced by DNA damage (11-13). Thus the cyclin B1 protein must be degraded in order to permit G2/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 G2/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 radiosensitivity 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 FC63G 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.

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 fixed in 70% ethanol and 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 BrdUl-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/m1 RNase (Sigma Chemical) in PBS for 30 min at room temperature. For both experiments twenty thousand events per sample were acquired using a FACScant device (Becton-Dickinson, Sunnyvale, CA, USA).

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 BrdU 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 BrdUl-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/m1 RNase (Sigma Chemical) in PBS for 30 min at room temperature. For both experiments twenty thousand events per sample were acquired using a FACScant device (Becton-Dickinson, Sunnyvale, CA, USA).

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
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 degraded, 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. β-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.
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 G2/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 G2/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 G2/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 G2/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 G2/M arrest. In 6 Gy-mitotane treated cells, without purvalanol-A administration, we observed a marked G2/M arrest. Six Gy-mitotane treatment induced an accumulation in the G2 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 G2 arrest was irreversible since, even 72 h after treatment, the G2 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 G2 arrest. Moreover, in 6 Gy-mitotane combined treatment any presence of the sub-G1 fraction,
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 G2/M fraction consistent with the presence of the sub-G1 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 G2/M block than in SW13 cells. In purvalanol-A-treated cells, we evidenced the presence of a sub-G1 peak (14 and 15% respectively, in H295R and SW13 cells), already at 24 h. The cell fraction in the sub-G1 peak reached about 18% in both cell lines concomitant with the depletion of the cells from G1 compartment (Fig. 5). This data suggest that purvalanol-A overcome G2/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.
they clearly show that ACC is not resistant to IR. Patients
protocols and despite the number of cases being rather small,
42% obtained with radiotherapy in ACC treatment (27-29).
mitotane (26).
ACC different therapeutic protocols have been proposed,
identifying a therapeutic range. Also in the later stages of
plasmatic dosage of the drug and the possibility of
ACC. At present its side effects are avoidable through the
recurrence-free survival in patients with radically resected
patients with ACC, that adjuvant mitotane may prolong
hormonal reduction in 75% of cases (24). Terzolo
It provides a clinical response in one-third of patients and an
the last decades and it still mainly relies on the use of mitotane.
treatment, which leads to complete healing in early stages of
ACC (23). Medical treatment has not substantially changed in
proteins, thus rendering these cells more susceptible to
radiation treatment by inhibiting MLH1 and MSH2
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 G2 phase.

Discussion

ACC is a rare cancer with a very poor prognosis. The therapeu-
tic 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 G2 delay of cell cycle
in H295R and SW13 adrenocortical cancer cells after IR and
mitotane exposure in combination. This result was accom-
panied 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 G2 checkpoint is a
critical cellular defence system that prevents mitotic entry in
response to DNA damage. Several mechanisms have been
implicated in G2 arrest such as the level of cyclin B1-Cdk1
activity blockage (11-14).
Conversely, the new synthesis and the cyclin B1 accumu-
lation during S and G2 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 G2/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 adrenocortical 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 G2/M transition,
suggesting that cyclin B1 stability is a key regulatory factor
in initiating G2 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

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 β-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.
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 (mismatch 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 sensitisises 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 G2/M phase, characterized by high cdc2 kinase activity.

Acknowledgements

This work was financed by Ateneo ‘06 research grant no. 8.1.1.2.16 from ‘Sapienza’ Università di Roma. The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

References


