Mitotane increases the radiotherapy inhibitory effect and induces G2-arrest in combined treatment on both H295R and SW13 adrenocortical cell lines

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Abstract

Mitotane, 1,1-dichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane (o,p′-DDD) is an agent with adrenotoxic effect, which is able to block cortisol synthesis. This drug and radiotherapy are used also in adrenal cancer treatment even if their biological action in this neoplasia remains unknown. We investigated the effects of o,p′-DDD and ionizing radiations (IR) on cell growth inhibition and cell cycle perturbation in H295R and SW13 adrenocortical cancer cells. Both cell lines were irradiated at a 6 Gy dose and were treated with o,p′-DDD 10−5 M separately and with IR/o,p′-DDD in combination. This combination treatment induced an irreversible inhibition of cell growth in both adrenocortical cancer cells. Cell cycle analysis showed that IR alone and IR/o,p′-DDD in combination induced the cell accumulation in the G2 phase. At 120 h after IR, the cells were able to recover the IR-induced G2 block while cells treated with IR/o,p′-DDD were still arrested in G2 phase. In order to study the molecular mechanism involved in the G2 irreversible arrest, we have considered the H295R cell line showing the highest inhibition of cell proliferation associated with a noteworthy G2 arrest. In these cells, cyclin B1 and Cdk2 proteins were examined by western blot and Cdk2 kinase activity measured by assay kit. The H295R cells treated with IR/o,p′-DDD shared an increase in cyclin B1 amount as the coimmunoprecipitation of Cdc2–cyclin B1 complex. The kinase activity also shows an increase in the treated cells with combination therapy. Moreover, in these cells, sequence analysis of p53 revealed a large deletion of exons 8 and 9. The same irreversible block on G2 phase, induced by IR/o,p′-DDD treatment, happened in H295R cells with restored wild-type p53 suggesting that this mechanism is not mediated by p53 pathway.

Introduction

Sporadic adrenocortical carcinoma (ACC) is an uncommon tumour that rarely occurs with synchronous bilateral adrenal involvement (Venkatesh et al. 1989). In advanced disease, highly individualized treatment includes surgical mass reduction, control of endocrine activity and alleviation of symptoms from local tumour growth (Schteingart 1992).

Mitotane, 1,1-dichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane (o,p′-DDD) is able to block cortisol synthesis by inhibiting 11β-hydroxylation and cholesterol chain cleavage, thus representing an effective agent in the treatment of the functional ACC (Trainer & Besser 1994, Beauregard et al. 2002). Nevertheless, the toxicity of o,p′-DDD has been a major limit to its suitability in the treatment of ACC patients (Schteingart et al. 1993). Some authors have described an excellent response to adjuvant radiotherapy in patients with ACC (Percarpio & Knowlton 1976, Markoe et al. 1991), while another study seems to suggest its use only as palliation in metastatic ACC (Nader et al. 1983). Furthermore, few data are
available due to the paucity of patients with different tumour stages (Bodie et al. 1989).

The p53 tumour suppressor gene has a crucial role in DNA repair and recognition of DNA lesions (Kastan et al. 1991, Di Leonardo et al. 1994). Its loss of function may lead to aberrant cell kinetics and tumour growth (Hollstein et al. 1991). Some authors demonstrated allelic losses at chromosome 17p containing p53 gene locus and at chromosome 13q containing RB gene locus in human ACC (Yano et al. 1989). Moreover, p53 gene mutations were also found in some ACC (Ohgaki et al. 1993, Hollstein et al. 1994, Reincke et al. 1994).

Aim of this study was to evaluate the antineoplastic effects of both radiotherapy and o,p′-DDD used individually or in combination either in H295R functional or SW13 non-functional adrenocortical cancer cells, derived from human ACC. The negative control was represented by Hs792(C).M, a human normal fibroblast cell line derived by similar embryologic sheet of adrenal cortical areas. We demonstrated that radiotherapy and o,p′-DDD in combination exert a significant antiproliferative effect on adrenocortical human cell lines, characterized by cell cycle arrest in G2 phase with an overexpression of cyclin B1 and a high Cdc2 kinase activity in the H295R cells. These results may suggest interesting implications in the treatment of ACC.

**Materials and methods**

**Cell culture and treatment**

Cell lines were supplied from the American Type Culture Collection (Rockville, MD, USA). The H295R steroid-synthesizing cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s medium (DMEM/HAM’S F-12), medium supplemented with penicillin/streptomycin 50 U/ml and enriched with a mixture of a insulin/transferrin/selemin and 10% NuSerum-I. The SW13 cells were grown in Leibovitz’s L-15 medium supplemented with 10% bovine serum and the Hs792(C).M cells in D-MEM supplemented with 1.5 g/l sodium bicarbonate and 10% bovine serum. All cell lines were irradiated by a Varian Clinac 600c/d 6MV photon beam. Scanditronix FC65G farmer ionization chamber was used to evaluate the beam properties in water and polymethylmethacrylate phantom. The cells irradiation was based on single irradiation doses of 6 Gy/min and analysed from 24 to 120 h. All experiments were repeated at least thrice and each experimental sample was seeded in triplicate.

Radiation treatment was given 24 h post-seeding and then the cells were treated with o,p′-DDD 10 μM (Sigma–Aldrich). The viable cells were counted using a haemocytometer by trypan blue exclusion.

The H295R cells were exposed to hydrocortisone (100 nM; Calabria et al. 2006) replacement after the combined treatment. Proliferation rate was performed until 120 h.

**Cortisol secretion**

Cortisol was determined in the H295R cell supernatant at different times after the treatment by radioimmuno metric assay (Bayer Corp).

**Cell cycle analysis**

Cell cycle was studied using both bromodeoxyuridine incorporation (BrdU; Sigma Chemical Co.) and propidium iodide (PI) staining. Both BrdU pulse-labelling and continuous-labelling experiments were carried out. The 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, the cells were continuously exposed for 50 h before analysis. After 30 min and after 50 h, the cells were harvested, washed once in PBS, fixed in 70% ethanol and stored at 4 °C before analysis. Then, the samples were incubated with mouse monoclonal antibody anti-BrdU (Roche Diagnostics) in a complete medium containing 20% fetal calf serum (FCS) and 0.06% Tween 20 (Calbiochem, San Diego, CA, USA) 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 represents 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, 20 000 events per sample were acquired using a FACScan cytofluorimeter (Becton Dickinson, Sunnyvale, CA, USA).

**Western blotting**

Cellular lysates were sonicated on ice, clarified by centrifugation at 20 000 g and stored at −80 °C. An aliquot of the cell lysates was used to evaluate the protein content by colorimetric assay. The subcellular fractions, nuclear and cytoplasmic proteins were prepared using the procedure of the Nuclear/Cytosol fractionation Kit (MBL international corporation Woburn, MA, USA). Seventy micrograms of each of the subcellular fractions were 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 and were then incubated with anti-cyclin B1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-Cdc2 (SC-54 Santa Cruz Biotechnology).

The cytoplasmic and nuclear fraction normalization were performed with anti-α tubulin (TU-02 Santa Cruz Biotechnology) and anti-histone 1 (AE4-Santa Cruz Biotechnology) respectively.

The treated and untreated H295R cells were incubated with the anti-p53 (DO-1, Santa Cruz Biotechnology) and anti-p53 (C-19, Santa Cruz Biotechnology) at the same concentration of 1:500. The visualization of the antigens was performed by enhanced chemiluminescent detection reagents.

Coimmunoprecipitation
The H295R cell pellets were resuspended in cell lysis solution at a low stringency (NP40 1%, leupeptin 1 µg/ml, pepstatin 1 µg/ml, aprotinin 2 µg/ml, phenylmethylsulphonylfluoride 0.2 mM, sodium fluoride 10 mM) and a protease inhibitor. Then, the samples were sonicated for 10 s (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, the supernatant was again incubated with the protein A and with cyclin B1 at 4 °C overnight. Immunocomplex were washed thrice in lysis solution at low stringency, resolved by SDS-PAGE. Proteins transferred to the filter were detected by Cdc2 horseradish peroxidase-linked secondary antibody and visualized by enhanced chemiluminescence reagent. The anti-cyclin B1 was used to normalize the immunocomplex.

Cyclin B1 kinase dependent assay
To measure the kinase activity of cyclin B1–Cdc2 in H295R cell lysate, CycLex Cdc2–cyclin B1 Kinase Assay Kit (CycLex Co. Ltd, Ina Nagano, Japan) was used. Phospho-specific monoclonal antibody used in this assay kit was able to recognize the phospho-threonine 376 residue in human Cdc7 that is phosphorylated only by Cdc2–cyclin B1 kinase. Quantitative measurement of Cdc2–cyclin B activity involved the incubation of Cdc2–cyclin B sample with substrate, either a natural or synthetic polypeptide in the presence of Mg2+ and 32P-labelled ATP. Filter papers were then washed to remove unincorporated radiolabel and the radioactivity counted by absorbance value.

PCR and sequence analysis of p53 gene
Total RNA was extracted from 1×10^6 H295R cells cultured in 100 mm dishes with 1 ml TRIZOL reagent and 200 µl chloroform/ml TRIZOL were used. The samples were centrifuged and then the isopropanol/ml TRIZOL was added to aqueous phase and incubated at room temperature for 10 min. Pellet was washed with Et-OH/ml TRIZOL and finally dissolved in 20 µl diethylpyrocarbonate (DEPC) water.

Genomic DNA of H295R cells was extracted by DNA isolation kit (Gentra System Qiagen, Chatsworth, CA, USA). PCR was performed in a total volume of 20 µl. The following primers of the p53 were used for PCR:

Ex5–Ex6 For 542
CTGCTAGATAGCAATGGTCTG
Ex7 Rev 704
TTGTAATGATGGTGACACGTC
Ex11 Rev 1183.

DNA was sequenced using the Big Dye Terminator Cycle Sequence Ready Reaction kit on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol.

Transient transfection of H295R cells
In H295R cell line, a transient transfection of the p53 plasmide construct was performed. Cells (3×10^5 in 100 mm dishes) were cotransfected the day after seeding with 4 µg pNDp53 and 2 µg pCMVEGFP-spectrin plasmids, mixed in Opti-MEM containing 25 µl LipofectAMINE reagent (Invitrogen) and incubated for 7 h according to the manufacturer’s instructions. Afterwards, the transfected cells were maintained in fresh growing medium for 24 h, exposed to single ionizing radiations (IR) dose (6 Gy), o,p’-DDD alone and in combination. After 72 h of treatment, the samples were processed for cell cycle analysis, as described below.

Results
Effects of IR and o,p’-DDD treatment in H295R and in SW13 cells
Kinetic characteristics of adrenocortical H295R and SW13 cells were investigated using BrdU incorporation and bivariate BrdU/DNA flow cytometric analysis (FCM). After 30-min pulse chase, the percentage of cells with active DNA replication detected by BrdU positive cells was about 45% in both cell lines. Cells were checked for 50 h in order to label the entire cell cycle population. As observed in Fig. 1, all cellular populations were completely labelled by BrdU. In fact, more than 90% of cells were BrdU-positive in both cell lines, indicating that all cells were in the S phase. This interval
of time allowed us to define 50 h as their potential doubling time (Tpot) in which DNA replication, mitosis and cell division were completed.

We exposed both adrenocortical cells to different radiation doses, 1-3-6-10 Gy, and examined the effects produced by the treatment on cell growth and cell cycle at different times after radiation exposure (24, 48, 72, 96 and 120 h). At each time, cells were harvested and counted using the trypan blue dye exclusion test. Figure 2A and B respectively show growth proliferation curves of H295R and SW13 cells treated with 1-3-6-10 IR Gy. IR doses of 1 and 3 Gy did not exert any significant antiproliferative effect within 120 h after treatment in both cell lines (about 7% respectively), whereas we observed a cell growth inhibition of about 70% at 72 h and continued 120 h after treatment (P < 0.01; Fig. 3A). In SW13 cells, it was about 55% at 120 h after treatment (P < 0.01; Fig. 3B), suggesting that cells were unable to recover from the IR-induced damage.

No relevant changes in cell growth were observed in o,p'-DDD-treated cell lines from 24 h until 120 h from treatment (Fig. 3A and B). Moreover, no significant effects were detected in the H295R cells treated with IR.

These data showed a significant and persistent inhibition of cell growth at the highest radiation dose (10 Gy). Conversely, at lower dose of 6 Gy, a fraction of the cell population seemed to be able to recover from the radiation-induced DNA damage. Non-toxic 6 Gy dose was selected to perform the experiments. Schteingart et al. (1993) reported that o,p'-DDD at 10 μM dose inhibits cortisol secretion with minimal effect on cell viability; therefore, we used this dose to evaluate the o,p'-DDD sensitivity on adrenocortical cells.

Proliferation of cells exposed to 6 Gy and adrenolytic o,p'-DDD in combination was inhibited to a greater extent than that observed after 6 Gy alone. This inhibitory effect was present in both cell lines. In particular, in H295R cells, it was about 70% after 72 h and continued 120 h after treatment (P < 0.01; Fig. 3A). In SW13 cells, it was about 55% at 120 h after treatment (P < 0.01; Fig. 3B), suggesting that cells were unable to recover from the IR-induced damage.

Figure 1 Representative cell cycle analysis after BrdUrd incorporation in the H295R and SW13 cells. Both cell lines were labelled with a 10 μM BrdU pulse of 30 min (a and c) and continuously labelled with BrdU for 50 h (b and d). Cells were considered BrdU-positive when detected above the line; the percentage of such cells is provided on the left. Representative of three different experiments with similar results.

Figure 2 Four different doses of ionizing radiations were tested on cell growth. No differences between 1 and 3 Gy doses on cell growth were detected. After 72 h of irradiation at 6 Gy dose, there was a 30% proliferation inhibition to (A) H295R cells and a 27% to (B) SW13 cells, the 10 Gy dose was toxic for the cell viability.
and o,p'-DDD after hydrocortisone replacement in culture medium (data not shown).

Finally, the same experiments performed on Hs792(C).M control cells did not affect their proliferation rate (Fig. 3C).

Steroid inhibition on H295R cells

H295R cells are able to secrete different steroid hormones (i.e. glucocorticoids, mineralocorticoids and adrenal androgens). To verify drug efficiency on inhibition of cortisol secretion, we measured their concentration in the culture medium. o,p'-DDD alone or in combination with 6 Gy inhibited hormone secretion of about 60% at 120 h after treatment ($P < 0.01$). No significant difference was observed in cortisol level in samples treated by 6 Gy alone (Fig. 4).

IR and o,p'-DDD in combination treatment induce arrest in G2 phase in H295R and SW13 cells

PI staining and FCM were employed to evaluate whether the o,p'-DDD alone or in combination with IR treatment induced cell cycle perturbations. The relative number of cells in each phase of the cell cycle was estimated from DNA content by cell Quest software analysis (Columbus, OH, USA).

As shown in Table 1, IR exposure induced an accumulation of the H295R cells in the G2 phase of cell cycle when compared with untreated cells and then evaluated 24 h after treatment (the G2 phase was 60 and 17% in 6 Gy treated and untreated H295R cells respectively; $P < 0.05$).

After 6 Gy exposure, in H295R-treated cells, the G2-phase percentage progressively decreased to 16% within 120 h from IR treatment and the G1 phase increased (69%). This analysis showed that the characteristic G2 block produced by IR treatment was completely recovered 120 h after treatment. IR and o,p'-DDD in combination induced a similar G2 accumulation (50 vs 17% in the control; $P < 0.05$) evaluated 24 h after treatment. However, a significant difference between two treatments was observed during the progression through the cell cycle phases ($P < 0.05$). It suggests that the cells were not able to recover the IR/o,p'-DDD-induced G2 block.

Also in SW13 cells, after 6 Gy exposure occurred a temporary G2 accumulation. Similar irreversible G2 arrest was observed in these cells when treated with 6 Gy/o,p'-DDD in combination. In fact, it induced a G2 arrest at 24 h (30 vs 7% in the control) and in the following times ($P < 0.05$; Table 2).

Adrenocortical cancer cells treated in combination were still blocked in the G2 phase after 120 h from treatment compared with IR exposure alone (67 vs 16% in H295R, $P < 0.05$; 27 vs 14% in SW13, $P < 0.05$), suggesting that prolonged G2 block could be responsible for increased IR sensitivity. No cell cycle perturbation was observed in both cell lines exposed to o,p'-DDD alone.

Similar results were obtained in Hs792(C).M control cells (data not shown) according to the data reported by Ceraline et al. (1997).
Table 1 Flow cytometric analysis of H295R cells

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<th>96 h</th>
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<td>G1 %</td>
<td>S %</td>
<td>G2 %</td>
<td>G1 %</td>
<td>S %</td>
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<td>G1 %</td>
<td>S %</td>
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<tr>
<td></td>
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<td>69±1.2</td>
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<tr>
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Cell cycle analysis after PI staining was performed at 24, 48, 72, 96 and 120 h from treatments. A 6 Gy dose induced a cell accumulation in the G2 phase of cell cycle after 24 h (60 vs 17% in control cells; \( P < 0.05 \)). In treated cells, G2 phase progressively decreased to the percentage of 16% within 120 h from IR treatment with progressive increase in the G1 phase (69%). IR and o,p'-DDD in combination induced a similar G2 accumulation (50%) evaluated 24 h after treatment and the cells were not able to recover the IR-induced G2 block until as 120 h (\( P < 0.05 \)). No significant variation in cell cycle was observed in the cells treated with o,p'-DDD alone.

Table 2 Flow cytometric analysis of SW13 cells

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<td>6 Gy + o,p'-DDD</td>
<td>55±0.1</td>
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Cell cycle analysis after PI staining was performed at 24, 48, 72, 96 and 120 h from treatment. A 6 Gy dose induced a cell accumulation in the G2 phase of cell cycle after 24 h (36 vs 7% in control cells; \( P < 0.05 \)). In treated cells, G2 phase progressively decreased to the percentage of 14% within 120 h from IR treatment with progressive increase in the G1 phase (74%). IR and o,p'-DDD in combination induced a similar G2 accumulation (30%) evaluated 24 h after treatment and the cells were not able to recover the IR-induced G2 block until as 120 h (\( P < 0.05 \)). No significant variation in cell cycle was observed in the cells treated with o,p'-DDD alone.
Cyclin B1 increased level after IR and o,p′-DDD combined treatment in H295R cells

H295R cells showing the highest inhibition of cell proliferation after IR/o,p′-DDD in combination were chosen in order to study the molecular mechanism involving the G2 irreversible arrest. Therefore, we analysed the expression of cyclin B1 in these cells. Western blot analysis of cyclin B1 was performed 24, 48 and 72 h after treatment. Cyclin B1 level increased significantly after IR and o,p′-DDD in combination compared with control cells, with a further increase of 1.5-fold after 24 h and 6.0-fold after 72 h of treatment. It was consistent with a marked G2-phase accumulation (66%). When 6 Gy IR was given to cells, a similar cyclin B1 increase of about 2.5-fold was observed after 24 h (Fig. 5A).

However, the cyclin B1 levels was decreased by about 1.5-fold at 72 h (Fig. 5A), and this reduction was associated with a marked G2 depletion (23%; Table 1).

Cdk1–cyclin B1 kinase activity in H295R cells

Since recent reports indicate that cells arrested in G2 can contain high levels of cyclin B1–Cdk1 associated kinase activity (Jin et al. 1996, Minemoto et al. 2003), we evaluated kinase activity by ELISA assay in H295R cells. First, we verified the formation of immunocomplex between cyclin B1 and Cdc2 proteins and found that cyclin B1 was able to bind Cdc2 protein as shown in Fig. 5B. A persistent increase of Cdk1-associated kinase activity (40% after 72 h) in combination treatment compared with control cells was detected after 24, 48 and 72 h according to Jin et al. (1996).

These results suggest that mitosis did not occur despite the apparent presence of abundant Cdk1 activity (Fig. 6). To confirm these data, we tested the localization of cyclin B1 in cellular compartments.

As shown in Fig. 7, there was a 1.5-fold cytoplasmic accumulation after 72 h of cyclin B1 protein only in cells treated with 6 Gy IR and o,p′-DDD in combination. These results demonstrated that the G2 block may be attributable to delayed progression of M phase with high Cdk1 activity, due to accumulation of the cyclin B1 protein preventing G2/M transition.

Role of the p53 on the G2/M transition in H295R cells

Since recently the role of p53 in G2 arrest has been reported (Jin et al. 1996, Minemoto et al. 2003), we characterized genomic sequence of p53 gene by direct sequencing of PCR-amplified products in H295R cells. As shown in Fig. 8A, electropherogram performed on forward and reverse sense revealed a homozygous deletion of exons 8–9 of the p53 gene. This region includes carboxyl terminus of p53 protein, one of the hallmarks of wild-type p53 for recognition and binding of damaged DNA (Reed et al. 1995, Zotechev et al. 2000).
In order to confirm the sequencing analysis data, a western blot experiment was performed. Extracts from whole untreated cells were tested using an antibody raised against amino acids mapping at C-terminus of p53 protein and an antibody against N-terminus of the protein. h-Panc, a cell line carrying a p53 point mutation, was used as positive control (Butz et al. 2003). The presence of domain at N-terminus of p53 protein was detected in both cell lines (Fig. 8B). The p53 carboxy terminus expression was found in h-Panc cells, whereas no specific signal was detected in H295R cells (Fig. 8C). Absence of signal in H295R cell line may explain large deletion occurring in these cells.

Finally, in order to directly address the role of p53 in the G2 arrest induced by IR/o,p'-DDD in combination, we sought to determine the effects of these treatments in the H295R adrenocortical cell line wild-type p53 restored. The p53 expression vector was transiently cotransfected with the green fluorescent protein (GFP)-spectrin expression plasmid in H295R cells and the DNA content profile of transfected cells was analysed by two-colour flow cytometry (Fig. 9). Gating out GFP-positive cells and analysing the cell cycle distribution of this population 72 h after 6 Gy IR and o,p'-DDD treatment used alone or in combination, we showed a similar G2 arrest (60%; Fig. 9, l) compared with the H295R p53 mutant cells (66%; Table 1) strongly suggesting that this mechanism is not mediated by p53 pathway.

Discussion

In this study, we evaluated different treatments on two types of adrenocortical cancer cell lines derived from human ACC in order to determine which one was able to induce an antineoplastic effect. We used IR at dose 6 Gy and o,p'-DDD at concentration of 10 μM alone or in combination. There is evidence supporting that radiation therapy is effective in the treatment of solid tumours, such as breast, prostate and ovarian cancers (Morton & Thomas 1994, Einhorn et al. 1999, Van der Steene et al. 2000). Moreover, in many types of cancer, it has been observed that adjuvant radiotherapy is associated with a long-term survival (De Crevoisier et al. 2004, Ragaz et al. 2005). All eukaryotic cells show cell cycle delay after the exposure to DNA-damaging agents (Concin et al. 2003, Marekova et al. 2003, Shimamura...
IR antiproliferative effects were studied in some cell lines. In fact, ovary and breast carcinoma cells and pituitary adenoma human cell lines have been described to respond to DNA damaging through cell cycle arrest inducing DNA repair pathways, or apoptotic process by activating effectors molecules of death (Kao et al. 2001, Iliakis et al. 2003, Nome et al. 2005). 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 paper, Allolio & Fassnacht (2006) affirm that radiotherapy might play a role as adjuvant therapy after surgery in patients with high risk of local recurrence. Actually, o,p'-DDD represents the main adrenolytic compound employed in the treatment of patients affected by ACC (Hahner & Fassnacht 2005). It is administered especially in order to reduce symptoms and clinical signs due to steroid excess. Unfortunately, its use is limited because of its toxicity and biochemical side effects on patients (Schteingart et al. 1993).

**Figure 8** Electropherogram of the p53 cDNA of the untreated H295R cells. Aliquots of the PCR-purified template DNA were sequenced by transcriptional sequencing. As shown, the deletion of exons 8 and 9 was detected (A) Western blot analysis of p53 protein derived from untreated cells H295R and h-Panc used as control for protein expression. Different antibodies were used to detect the protein in both cells lines: the p-53 N-terminal protein was detected in both cell lines (B) and p-53 C-terminal was detected only for the cell line h-Panc. It was absent in the H295R cell line (C).

**Figure 9** Cotransfection analysis of the wild-type p53 expression vector with the green fluorescent protein (GFP)-spectrin expression plasmid in H295R cells. The transfected cells were exposed to single IR dose (6 Gy) and o,p'-DDD alone and in combination. The analysis of positive cells after 6 Gy IR and o,p'-DDD treatments in combination showed a G2 arrest. FL1-H, green height fluorescence intensity; FL2-H, red height fluorescence intensity.
In view of these data, we examined the different antineoplastic effects on adrenocortical cancer cells using IR and o,p'-DDD alone or in combination. In this report, o,p'-DDD compound did not significantly influence cell growth and no perturbation of cell cycle was detected by FACS analysis in both cell lines. Moreover, in H295R adrenocortical functional cells, o,p'-DDD was able to inhibit cortisol secretion at a concentration of 10 μM (Fig. 4), as reported by several authors (Trainer & Besser 1994, Beauregard 1996, Varshavsky 1997). The 6 Gy IR determined, in both cellular models, a significant growth inhibition in treated cells with respect to control (P < 0.01). This inhibitory effect was partially lost during the following hours decreasing to 13% in H295R cells and to 20% in SW13 cells after 120 h. Nevertheless, when IR and o,p'-DDD were used in combination in both adrenocortical cancer cells, a lasting growth arrest of 70% at 120 h after treatment occurred in H295R and 55% at 120 h after treatment in SW13 cells.

These data for the first time support the effectiveness of radiotherapy on growth inhibition in adrenocortical cancer cells and strongly suggest the adjuvant role of mitotane in inducing a persistent cell cycle G2 block. The mechanism of action of mitotane drug in this combinatorial effect is still unclear. It appears adrenocortical cancer cells specific. Indeed, the effects demonstrated on H295R and SW13 cell lines are completely discordant with those observed in Hs792(C).M control cells. Moreover, it seems not to be cortisol related. In fact, the replacement of hydrocortisone in H295R cells culture medium did not exert a significant modification in cell proliferation. These data suggest that this drug inhibits the steroidogenic process in synthesizing adrenocortical cancer cell (H295R) and induces an irreversible G2 arrest in radiotherapy-combined treatment in both functional (H295R) and non-functional (SW13) adrenocortical cancer models. Several hypotheses could explain the persistent cell arrest at G2/M transition. The presence of a mechanism that is able to prevent the degradation of cyclin B1, involving an increase of Cdk1–cyclin B1 stability complex, cannot be excluded. Another possibility is the presence of a different cell cycle checkpoint mechanism that might control mitotic entry/exit. Finally, M-phase delay may be due to the inhibition of the ubiquitin–proteasome pathway mechanism for protein turnover and cell cycle control (Ciechanover 1994, Sudakin et al. 1995, Hochstrasser 1996, Varshavsky 1997). The G2-phase accumulation after 6 Gy IR and o,p'-DDD in combination, observed in H295R cell line, was characterized by overexpression of cyclin B1. It was associated with high Cdk1 kinase activity according to Jin et al. (1996) who showed a significant G2 delay associated with high Cdk1-associated kinase activity. Moreover, the cytoplasmic cyclin B1 increase occurred only after the treatment with IR and o,p'-DDD in combination. These data are in good agreement with the report by Smeets et al. (1994). It suggests a higher cytoplasmic amount of cyclin B1 in cells arrested in G2 by DNA damage.

Minemoto et al. (2003) reported an overexpression of cyclin B1 and high kinase activity in the lung adenocarcinoma cells subjected to genotoxic stress. This can be correlated to a ‘p53-dependent mechanism’, as p53 protein is up-regulated by genome stress and maintains genomic stability through the induction of cell cycle arrest. Furthermore, just as cyclin B1 and Cdk1, p53 negatively regulates the activity of genes that control the onset of mitosis (Krause et al. 2000, Dan & Yamori 2001). Cells with normal p53 levels arrest in G1 and G2, whereas cells that have lost p53 activity as a result of mutation arrest exclusively in G2 (Di Leonardo et al. 1994).

H295R cells showed a whole deletion of exons 8 and 9, localized in the proximity of the carboxy-terminal region, which includes nuclear export signal (NES), Oligo (tetramerization domain that includes a NES) and nuclear localization signal of p53 protein. Loss of these regions confers functional inactivity to the protein (Reed et al. 1995, Zotchev et al. 2000). In this cell line, G2 arrest, induced by IR and o,p'-DDD, is not mediated by p53 pathway since the G2 block persists when restoring wild-type p53.

In summary, these results show that IR and o,p'-DDD in combination inhibit cell growth and induce an irreversible block of the cell cycle in G2 phase in functional (H295R) and in non-functional (SW13) adrenocortical cancer cells. This block is supported by a characteristic perturbation of G2-checkpoint mechanism, involving Cdk1–cyclin B complex and Cdk1-increased activity in H295R cell line with abolished p53 function.

These findings indicate that o,p'-DDD increases the antitumoural effect of radiotherapy and suggest that IR and o,p'-DDD in combination could be considered as a promising therapeutic approach in the treatment for ACC.

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References

Beauregard C, Dickstein G & Lacroix A 2002 Classic and recent etiologist of Cushing’s syndromes diagnosis and therapy. Treatments in Endocrinology 1 79–94.


Zotchev SB, Protopenova M & Selivanova G 2000 p53 C-terminal interaction with DNA ends and gaps has opposing effect on specific DNA binding by the core. *Nucleic Acids Research* 28 4005–4012.