



Mitotane-driven apoptosis in adrenocortical carcinoma: A molecular insight

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Abstract

Aim: The aim of this study was to investigate the responses of antiapoptotic Bcl-2 and proapoptotic Bid genes to Mitotane and other chemotherapy drugs in Adrenocortical Carcinoma (ACC) cells. The purpose was to understand the effects of these chemotherapy drugs on apoptosis-related genes in ACC and to identify potential treatment pathways.

Materials and Methods: The study involved the use of ACC cells to assess gene expressions in response to treatment with Mitotane, Etoposide, and Cisplatin. Gene expression levels of Bcl-2 and Bid were measured after drug exposure, providing insight into the modulation of apoptosis pathways by Rt-qPCR.

Results: The results demonstrated that Mitotane notably affected the expression of the proapoptotic Bid gene in ACC cells, promoting apoptosis. Cisplatin increased the expression of the antiapoptotic Bcl-2 gene and decreased the expression of the proapoptotic Bid gene compared to Mitotane.

Conclusion: This study showed that Mitotane, like other chemotherapy drugs, affects the expression of key apoptosis-related genes in ACC cells. Mitotane significantly affected the proapoptotic Bid gene, indicating its potential as a treatment option for ACC. These findings suggest that Mitotane affects ACC cells, highlighting its importance in ACC therapy.



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Introduction

Adrenocortical carcinoma (ACC) is an infrequent but aggressive tumor, with an annual incidence of only 1 to 2 cases per million individuals [1]. The prognosis for ACC is exceedingly grim, with an overall 5-year survival rate ranging from only 15% to 44% [2]. Diagnosing ACC poses significant challenges owing to its clinical progression. The disease's stage at the time of diagnosis plays a pivotal role in determining prognosis. When detected at stage I, the survival rate can be as high as 80%. Conversely, if the diagnosis occurs at stage IV, the survival rate plummets to a mere 13% [3]. Metastasis represents a prevalent challenge in ACC cases. Despite surgical interventions being a part of the treatment regimen for ACC, the tendency for recurrence necessitates the application of chemotherapy protocols [4].

In the treatment of ACC, established chemotherapy agents including Etoposide, Cisplatin, and Doxorubicin, alongside Mitotane, classified as an orphan drug, are com-

monly employed. The administration of these chemotherapy drugs, including Mitotane, is typically tailored to the patient's clinical progression. Mitotane may be administered either as a standalone treatment or in combination with other chemotherapy drugs [5]. Mitotane is an adrenocytolytic medication that has received approval from both the US Food and Drug Administration and the European Medicines Agency for the treatment of ACC [6]. Its origins trace back approximately 70 years ago to the insecticide DDT, and it functions as a selectively destructive agent specifically targeting adrenocortical tissue [7].

The established threshold for plasma mitotane concentration in ACC patients is 20 mg/L [8]. Elevated plasma concentrations exceeding this threshold can lead to adverse effects on the gastrointestinal and central nervous systems [9]. Mitotane has demonstrated an increase in patient survival in just 33% of cases [10]. The impact of Mitotane on ACC cells remains relatively obscure, and its influence on cell death is not well-defined [3].

Proteins belonging to the Bcl-2 family are pivotal in regulating cell death [11]. This gene family encompasses both antiapoptotic and proapoptotic members. Bcl-2 primarily

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enhances cell survival and proliferation. In essence, Bcl-2 augments the overall cell count by averting cell death rather than accelerating the rate of cell division. Considering that the persistence of these cells without undergoing programmed cell death can lead to cancer, it is hypothesized that Bcl-2 inhibits certain types of cell death [12]. While the majority of Bcl-2 homologues function to inhibit cell death, certain Bcl-2 homologues, such as Bax, Bak, and Bid, are categorized as proapoptotic agents [13]. Despite its membership in the Bcl-2 family, Bid is considered a proapoptotic gene. Bid is from the BH3 homologous group of Bcl-2. Both pro- and antiapoptotic Bcl-2 family proteins have been shown to have additional functions necessary for the normal physiology of healthy cells [14].

These unconventional functions are improbable to be entirely accounted for by the conventional apoptosis-regulating activities, wherein anti-apoptotic Bcl-2 proteins directly interact and inhibit proapoptotic Bcl-2 family proteins to regulate the release of cytochrome-c from mitochondria in the intrinsic apoptotic pathway [12]. Bid assumes a pivotal role in facilitating cross-communication between the extrinsic pathway (involving extracellular ligand binding to cell surface death receptors) and the endogenous apoptosis pathways [15]. The impact of mitotane and other chemotherapy agents (such as Etoposide, doxorubicin, and cisplatin) employed in ACC treatment on the apoptosis of ACC cells remains uncertain.

In this study, these drugs were applied to H295 human ACC cell line, and their effects on Bcl-2 and Bid apoptosis genes were examined.

Materials and Methods

Cell lines and culture conditions

In the study, the NCI-H295R cell line which is human ACC cells (ATCC® CRL-2128™) was used. The cells were cultured in 75 cm² culture flasks at 37°C in a humidified incubator at 5% CO₂. For H295 cells, the culture medium consisted of DMEM/F12K medium, supplemented with 5% FCS, penicillin (1x10⁵ U/l), and L-glutamine (2 mmol/l). Cells were harvested with trypsin (0.05%)–EDTA (0.53 mM). Cell viability always exceeded 95%.

Drugs and chemicals

Mitotane (Cat. No. SML 1885) was procured from Sigma, while other chemotherapy drugs, including Etoposide (Cat. No. E1383), Cisplatin (Cat. No. P4394), and Doxorubicin (Cat. No. D1515), were also obtained from Sigma. Mitotane was dissolved in ethanol to create a 10-2M stock solution. Etoposide was prepared as a dimethylsulfoxide (DMSO) 10⁻² M stock solution. Cisplatin and Doxorubicin were dissolved in distilled water to generate 10⁻²M stock solutions. Mitotane and Etoposide stock solutions were stored at -20°C, while Cisplatin and Doxorubicin stock solutions were stored at +4°C.

Cell proliferation assay

For the assessment of total DNA content, cells were seeded in 1 ml of medium in plates at a density sufficient to achieve a 75–80% cell confluence by the conclusion of the

experiment. Subsequently, after twenty-four hours, Mitotane, Cisplatin, Etoposide, and Doxorubicin were introduced to the cells in the plates. Following 72 hours of treatment, the cells were collected for DNA measurement. The total DNA content within the cells was quantified utilizing the Hoechst 33258 reagent [16].

Quantitative RT-PCR

The expression levels of Bid and Bcl2 genes, along with Actin β as a housekeeping gene, in H295 cells were assessed through quantitative RT-PCR. Total RNA was extracted from H295 cells using the High Pure RNA Tissue kit (Roche). Subsequently, cDNA synthesis was conducted using the Transcriptor first Strand cDNA Synthesis kit. Real-time PCR was employed for the quantification of these genes, utilizing the Roche LightCycler® 480 system (Germany). The real-time PCR protocol consisted of an initial incubation at 95°C for 5 minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 10 seconds.

qPCR array data analysis

The Ct values for the genes were computed using the Roche LightCycler® 480 system. Expression values were then determined utilizing the $2^{-\Delta\Delta C_t}$ method, with calculations based on the housekeeping gene. Expression levels were assessed in the control group without medication and compared to the medicated control group. The gene expression levels were quantified in terms of fold change.

Statistical analysis

GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) was used for statistical analysis. The fifty percent growth-inhibitory concentrations (IC₅₀) were determined utilizing a nonlinear regression curve-fitting program. Experiments were repeated at least for times (n=4). Initial comparative statistical assessments among groups were carried out using the one-way ANOVA test (TUKEY). Statistical significance was defined as p values less than 0.05 (*p<0.05, **p<0.01, ***p<0.001).

Results

Effects of Mitotane, Etoposide, Cisplatin, and Doxorubicin on cell growth of the H295 cell line

Mitotane, Cisplatin, Etoposide, and Doxorubicin were administered to H295 cells and their cytotoxic effects were investigated. After 72 hours of incubation, the IC₅₀ value of Mitotane in H295 cells was calculated as 10 μ M (Figure 1.A). Figure 1.B shows the cell proliferation graph after 72 hours of H295 cells treated with different concentrations of Mitotane. Accordingly, after 72 hours of incubation, the proliferation of the cells treated with 100 μ M Mitotane decreased statistically significantly when compared to the control group (p<0.001). There is a statistically significant difference between the proliferation of the cells treated with 10 μ M Mitotane and the control group after 72 hours (p<0.05). High concentration Mitotane treated cells have a statistically significant decreased cell proliferation when compared to low concentration Mitotane treated cells.

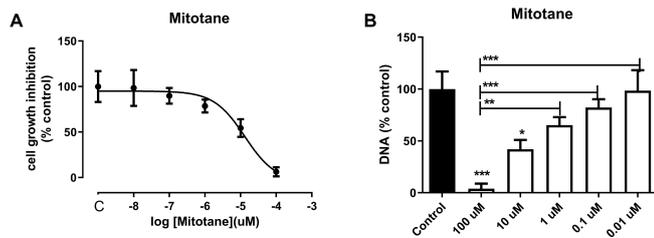


Figure 1. (A) Dose-time dependent effect of Mitotane treatment after 72h treatment in H295 cells. (B) Effect of Mitotane treatment on proliferation after 72h incubation time in H295 cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

After 72 hours of incubation, the IC_{50} value of Cisplatin for H295 cells was calculated as 10 μM (Figure 2.A). The cell proliferation graph of H295 cells after 72 hours of incubation in which different concentrations of Cisplatin were applied is shown in Figure 2.B. Accordingly, after 72 hours of incubation time, the proliferation of the cells treated with 100 μM Cisplatin decreased statistically significantly when compared to the control group ($p < 0.05$).

After 72 hours of incubation time, there is a statistically significant difference between the proliferation of the cells treated with 10 μM Cisplatin and the control group ($p < 0.05$). Proliferation rates of Cisplatin-treated H295 cells decreased at high concentrations of cisplatin when compared to low concentrations of cisplatin. After 72 hours of incubation, the IC_{50} value of Etoposide for H295 cells was calculated as 3 μM (Figure 2.C). When the cell proliferation of H295 cells treated with different concentrations of Etoposide was examined after 72 hours of incubation, there was a statistically significant decrease in the proliferation of the cells treated with 10 μM Etoposide when compared to the control group ($p < 0.05$) (Figure 2.D).

The calculated IC_{50} value of H295 cells treated with different concentrations of Doxorubicin after 72 hours was 1 μM (Figure 2.E). The effect of Doxorubicin on ACC cells decreases at low concentrations. Doxorubicin applied at a concentration of 10 μM was shown to suppress H295 cells by 96% (Figure 2.F).

Expression of the Bid and Bcl-2 genes in the H295 cell line

It was determined how the expression of the Bid and Bcl-2 genes, which are active in the apoptosis pathway, is altered by drugs in H295 cells.

The Bid gene was expressed 2.6 times more in H295 cells treated with mitotane when compared to the control group, and there was a statistically significant difference between them ($p < 0.05$). Mitotane downregulated the Bcl-2 gene expression level in H295 cells and was 0.6-fold compared to the control (Figure 3.A). Cisplatin overexpressed the Bid gene in H295 cells ($p < 0.001$). Cisplatin increased the Bid gene expression level 3.8 fold compared to the control group, while it increased the Bcl-2 gene expression level 2 times compared to the control group (Figure 3.B). The effect of Etoposide on Bid and Bcl-2 genes in H295

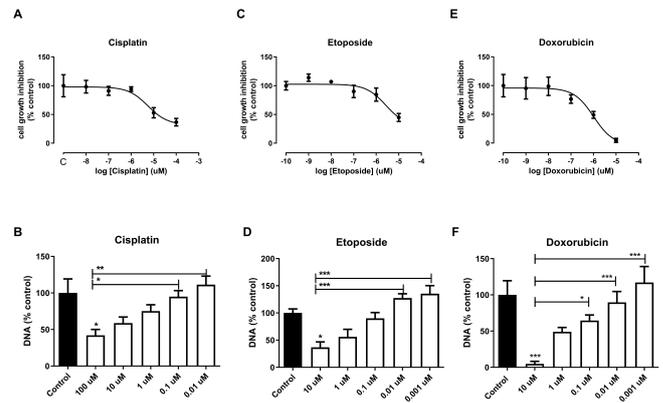


Figure 2. (A) Dose-time dependent effect of Cisplatin treatment after 72h treatment in H295 cells. (B) Effect of Cisplatin treatment on proliferation after 72h incubation time in H295 cells. (C) Dose-time dependent effect of Etoposide treatment after 72h treatment in H295 cells. (D) Effect of Etoposide treatment on proliferation after 72h incubation time in H295 cells. (E) Dose-time dependent effect of Doxorubicin treatment after 72h treatment in H295 cells. (F) Effect of Doxorubicin treatment on proliferation after 72h incubation time in H295 cells. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

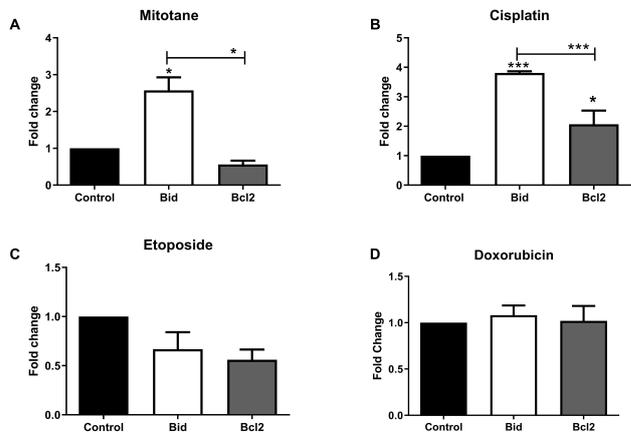


Figure 3. Bid and Bcl2 Gene Expressions in H295 Cells in Response to Drug Administration: (A) Mitotane, (B) Cisplatin, (C) Etoposide, (D) Doxorubicin. Expression levels were quantified using the $2^{-\Delta\Delta Ct}$ analysis method, indicating statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

cells was more different from other drugs. Etoposide decreased the expression levels of Bid and Bcl-2 genes by 0,6 and 0,5 fold, respectively (Figure 3.C). Doxorubicin did not change the expression levels of Bid and Bcl-2 in H295 cells compared to the control group (Figure 3.D).

The effect of Cisplatin, Etoposide, and Doxorubicin drugs on gene expressions according to Mitotane was evaluated (Figure 4). The Ct values of genes affected by these drugs

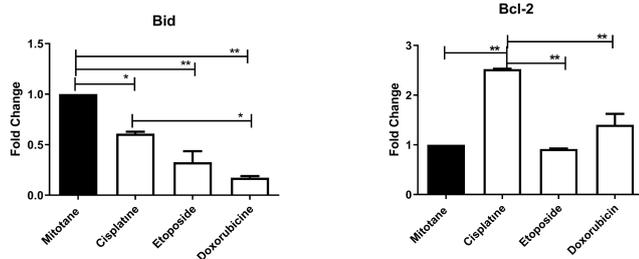


Figure 4. Changes in the expressions of BID and BCL-2 genes according to Mitotane when Cisplatin, Etoposide, and Doxorubicin are administered to H295 cells.*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

were calculated according to Mitotane. While the effect of Mitotane on the Bid expression level was 1-fold according to $2^{-\Delta\Delta C_t}$ analysis, Cisplatin suppressed the Bid expression level 0.6-fold compared to Mitotane ($p < 0.05$). Etoposide suppressed the Bid expression level 0.3 times compared to Mitotane ($p < 0.05$) (Figure 4.A). Cisplatin increased the Bcl-2 gene expression level 2.5 fold compared to Mitotane ($p < 0.001$). Etoposide suppressed Bcl-2 gene expression level by approximately 0.9-fold. Doxorubicin suppressed Bid expression level 0.1-fold compared to Mitotane. It was also almost as effective as Mitotane at the Bcl-2 gene expression level (Figure 4.B).

Discussion

In this study, the responses of antiapoptotic Bcl-2 and proapoptotic Bid genes to Mitotane and other chemotherapeutic drugs in ACC cells were investigated. Defective expression of genes in the proapoptotic Bcl-2 family occurs with the loss of P53 function. There is a strong association between P53 and Bcl-2 genes [17]. Furthermore, when the genomic profile of ACC was examined, mutations in the P53 gene were detected in 14% of cases [18]. Besides, the Bid gene is one of the direct targets of the P53 gene [19]. Although it is thought that the Bcl-2 gene may be defective due to P53 gene mutation in ACC cells, this study showed that Mitotane did not have a significant effect on the antiapoptotic Bcl-2 gene.

Antiapoptotic Bcl-2-family proteins can suppress cell death caused by cytotoxic anticancer drugs [17]. There are several assumptions about how mitotane affects the apoptosis of ACC cells, but there is no clear information. It is thought that Mitotane acts on ACC cell apoptosis through the mitochondrial pathway [3,7]. The intrinsic signalling pathway triggers cell death through mitochondrial outer membrane permeation (MOMP). MOMP enables the release of pro-apoptotic proteins (cytochrome c and other intermembrane space proteins) [20,21]. MOMP is regulated by members of the Bcl-2 family [22]. MOMP is only activated when Bcl-2 family proteins are inhibited and Bax or Bak are activated by activator BH3 proteins [23]. Bcl-2 expression levels have been found to be high in ACC [24].

In this study, Mitotane increased the proapoptotic Bid gene expression levels in ACC cells. Etoposide slightly sup-

pressed the Bcl-2 and Bid genes expression levels compared to the control group. Cisplatin overexpressed the Bid gene, while slightly increasing the expression of the Bcl-2 gene. Activation of Caspase 8 results in the activation of the Bid gene [25]. Caspases are a family of cysteine proteases that play a crucial role in apoptosis [26]. Caspase 8 also plays a role in the extrinsic apoptosis pathway [27]. Activation of Caspase 8 requires stimulation of FAS, TRAIL and TNF α receptors [28]. It has been shown that in ACC cells, Fas expression decreased while FasL expression increased compared to normal adrenal glands [29]. When TNF α is stimulated, FADD and then Caspase 8 are activated [28]. Although the TNF α gene is not expressed in ACC, tumor necrosis factor-alpha-induced protein 3 (TNFAIP3), which regulates TNF α , is also overexpressed in ACC compared to the normal adrenal gland [30]. However, serum levels of TNF α were particularly higher in patients with ACC and aldosterones [31].

Conclusion

In conclusion, this study has shown that the Bcl-2 and Bid genes, which have an important role in the apoptosis pathway in ACC cells, are affected by Mitotane and other chemotherapy drugs. It has been shown that Mitotane is more effective on the Bid gene, which enables ACC cells to undergo apoptosis. It has been observed that the effect of potent chemotherapy drugs such as Cisplatin and Etoposide on these genes is more uncertain than Mitotane. Cisplatin increased the expression of Bcl-2, which is an antiapoptotic gene while decreasing the expression of the proapoptotic gene Bid when compared to Mitotane. At the same time, it has been slightly determined that mitotane can enable ACC cells to undergo apoptosis not only through a single apoptotic pathway, but also through both extrinsic and intrinsic pathways.

Ethical approval

It is a study that does not require an ethics committee decision.

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