Turk J Pharm Sci 2018;15(3):364-369
DOI: 10.4274/tjps.94695

ORIGINAL ARTICLE

The Apoptotic and Anti-apoptotic Effects of Pendimethalin and Trifluralin on A549 Cells *In Vitro*

Pendimetalin ve Trifluralinin Apopitotik ve Anti-Apopitotik Etkilerinin A549 Hücrelerinde *İn Vitro* Değerlendirilmesi

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ABSTRACT

Objectives: Pendimethalin and trifluralin are commonly used in many countries to control broadleaf weeds and grassy weed species because of their inhibitor effects on growth and cell division. In this study, we examined the apoptotic and anti-apoptotic potentials of pendimethalin and trifluralin on A549 human non-small lung cancer cells with several concentrations *in vitro*.

Materials and Methods: The expression levels of apoptosis-related genes *BCL-2, BAX, CAS3, CAS9, P53, BIRC*, and *PPIA* were examined using quantitative RT-PCR after 24 h treatment of 1, 10, 50, 100 and 500 µM pendimethalin and trifluralin.

Results: The effects of pendimethalin were found more repressive than trifluralin on all studied concentrations. Twenty-four hours' exposure with 100 µM pendimethalin and trifluralin altered the gene expressions, suppressing apoptosis and allowing cancer cells to grow and proliferate.

Conclusion: Care should be taken not to exceed the permissible values and residue limits in food during pendimethalin and trifluralin use in order to reduce the possible carcinogenic effects on humans.

Key words: Pendimethalin, trifluralin, apoptosis, A549, gene expressions

ÖZ

Amaç: Pendimetalin ve trifluralin birçok ülkede, büyüme ve hücre bölünmesi üzerindeki inhibitör etkileri nedeniyle, geniş yapraklı yabani otları ve çimenli ot türlerini kontrol etmek amacıyla yaygın şekilde kullanılmaktadır. Bu çalışmada, pendimetalin ve trifluralinin apoptotik ve anti-apoptotik potansiyelleri, A549 insan küçük olmayan akciğer kanseri hücreleri üzerinde çeşitli konsantrasyonlarda *in vitro* incelendi.

Gereç ve Yöntemler: Apoptoz ile ilişkili genler *BCL-2, BAX, CAS3, CAS9, P53, BIRC* ve *PPIA*'nın ekspresyon seviyeleri, 24 saat 1, 10, 50, 100 ve 500 µM pendimetalin ve trifluralin uygulamasından sonra kantitatif RT-PCR ile incelendi.

Bulgular: Çalışılan tüm konsantrasyonlarda pendimetalinin etkileri trifluralinin etkilerine kıyasla daha fazla baskılayıcı bulundu. 100 µM pendimetalin ve trifluraline 24 saat boyunca maruz bırakılan hücrelerde gen ifadesi, apoptozu baskılayacak ve kanser hücrelerinin büyüme ve çoğalmasına yol açacak şekilde değişikliğe uğradı.

Sonuç: Pendimethalin ve trifluralinin insanlar üzerindeki olası kanserojenik etkilerini azaltabilmek için, kullanımları sırasında izin verilen değerlerin ve gıdalar üzerindeki kalıntı limitlerinin aşılmamasına dikkat edilmelidir.

Anahtar kelimeler: Pendimetalin, trifluralin, apopitoz, A549, gen ifadesi

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Received: 20.07.2017, **Accepted:** 17.08.2017

INTRODUCTION

Dinitroaniline herbicides were first discovered when dye and dye chemical intermediates were being evaluated. Chemicals in the dinitroaniline herbicide family essentially have a bright yellow color depending on the two nitro groups of the phenyl ring. They are generally referred to as "yellow compounds". The most important and the first herbicide in the dinitroaniline family is trifluralin, which became known in 1963.[1] Dinitroaniline herbicides are separated into two groups as methylaniline herbicides and sulfonyllanine herbicides. Pendimethalin and trifluralin are herbicidal compounds in the group of methylaniline.[2] Herbicides such as pendimethalin and trifluralin are used to control broadleaf weeds and grassy weed species in cabbage, celery, corn, cotton, garlic, lettuce, radish, rice, sorghum, tobacco, brassicas, carrots, cereals, citrus, onions, peas, peanuts, pome fruits, potatoes, soybeans, stone fruits, and tomatoes. Both herbicide compounds are also used in Turkey. Pendimethalin is used for growing apples, walnuts, hazelnuts, peanuts, potatoes, soy, citrus, grapes and asiatic seeds; trifluralin is used for growing cotton, soybean, sunflower seeds, sugar cane, citrus, tomatoes, peppers, onions, aubergine beans, carrots, cumin, and sesame in Turkey.[3]

Dinitroaniline group herbicide compounds pendimethalin and trifluralin can cause nitrosamine synthesis in animals and humans. Nitrozamines are highly reactive, harmful chemical species. They can act as carcinogenic substances by removing amino groups from the nucleotide bases of DNA. At the same time, they can act as toxic alkylating agents.[2] For these reasons, cancer is the suspected health effect and the risk of dietary exposure to pendimethalin and trifluralin. Pendimethalin and trifluralin are also present as contaminants in soil, ground water, surface water, and air because of the widespread use of various formulations.[4] Pendimethalin is classified as a slightly toxic compound (class 3) to mammals. Trifluralin has no acute toxicity on oral, dermal, and ocular exposures to mammals; although it is highly toxic to cold and warm water aquatic organisms as reported by the United States Environmental Protection Agency. Pendimethalin and trifluralin have also been classified as a group C-possible human carcinogen.[5]

Apoptosis, programmed cell death, is defined by important morphologic changes; blebbing, chromatin condensation, nuclear fragmentation, and cell shrinkage. It is an essential process for normal development and also related to chronic diseases with various pathologic situations such as cardiovascular, immunologic, neurodegenerative diseases, and cancer.[6]

Apoptotic mechanism is activated with many biochemicals, the best-defined pathway factors are caspases.[7] *CAS3* is the main protease in the cell death process; *CAS6* and *CAS7* also contribute to the coordination of apoptosis. Thus, these three caspases are known the 'executioner caspases'. In addition, *CAS8* and *CAS9* play a role in the initiation step.[8] When the initiative caspases activate the executioner caspases, the apoptotic process gets started with other enzyme activations.[9] The apoptosis process continues on the extrinsic or intrinsic (mitochondrial) pathway and results in cell death.[10] As well being a programmed process, apoptosis can occur after different kinds of irritations, such as radiation, anticancer drugs that cause DNA damage, and deprivation of cytokines that provide survival signals.[11]

In addition to enzymatic changes, the apoptotic pathway is directly related to some gene expressions. As an example, *BCL-2* family proteins control the intrinsic pathway on the antiapoptotic side; however, *BAX* and BAK proteins are promoters of cell death.[12,13] One of the most important proteins at the cell cycle checkpoint is the *P53* tumor suppressor protein, which can be activated by DNA damage, hypoxia, and apoptosis.[14]

Pesticides are known to lead cells to apoptosis in both the intrinsic and extrinsic pathways.[15-17] The compounds mainly enhance mitochondrial oxidative stress mediators and activate the cytochrome-C pathway, resulting in intrinsic apoptosis.[18,19] Dinitroaniline herbicides are used as weed controllers. Their mechanisms of action are based on cell division and decreasing cell elongation and growth with mitotic disruption during mitosis.[20,21]

In this study, we measured the expression levels of *P53*, *BAX, BCL-2, CAS3, CAS9, BIRC*, and *PPIA* (housekeeping) genes related to apoptosis on A549 human lung carcinoma cells after exposure to pendimethalin and trifluralin, which are two commonly used dinitroaniline herbicides.

MATERIALS AND METHODS

Solution preparation

Pendimethalin is highly soluble in oil and organic solvents.[22] The solution was prepared in a dimethyl sulfoxide (DMSO):olive oil (1:3, v/v) mix. Trifluralin is soluble in organic solvents and less soluble in water.[23] The trifluralin solution was prepared in PBS (1% DMSO).

Dulbecco's Modified Eagle's medium with 10% fetal calf serum and a 1% penicillin-streptomycin mixture were used as the cell culture medium.

Cell culture and treatment

A549 cells were cultured in a 25-cm^2 cell culture flask and transferred to a 75-cm^2 flask after 24 h under the conditions of 5% CO_2 and 37°C. After 24 hours, the cells were harvested and transferred to 6-well plates as 10,000 cell/2 mL medium of each. Cell counts were performed using Tripan blue (0.4% w/v in distilled water) in a Neubauer Chamber. One day later, when the cell count multiplied 2 folds and reached 20,000/well, pendimethalin and trifluralin solutions were added to the wells, the final concentrations were 1, 10, 50, 100 and 500 µM. These concentrations were chosen according to their 50% inhibitory concentration (IC_{50}) and toxicity levels.[24-27] The cells were incubated for 24 hours and harvested from the wells and centrifuged at 1200 rpm for 5 min.

RNA isolation, cDNA synthesis and gene expression

RNA isolation were performed using an RNeasy Mini Kit, QIAGEN in accordance with the manufacturer's instructions. In brief, after centrifugation, the cell suspension was filtrated

from the gDNA eliminator column, then transferred and attached to the RNeasy spin column and washed with the solutions as instructed.

The amount and quality of the eliminated RNA samples were measured using Maestrogen Nanodrop. For this measurement, 1 µL of the sample was loaded to the base portion fiber terminal. All the samples' OD 260/280 ratios were found in the range of 1.6-1.8.

The cDNA synthesis was performed from the RNA samples with RT² First Strand Kit, QIAGEN as per the manufacturer's instructions. The RNA samples were denaturized at 42˚C for 5 min in a real-time quantitative reverse transcription polyeramse chain reaction (qRT-PCR) device. The samples were placed on a cold surface to protect the linearity. Then reverse-transcription enzymes were added and the cDNA synthesis process was performed at 42˚C for 15 minutes and 90˚C for 5 minutes. Newly synthesized cDNA samples were stored at -20˚C. The PCR primers used in this study are listed in Table 1.

To measure the expression levels of apoptosis-related genes, cDNA samples were mixed with RT² qPCR primers (*BCL-2, BAX, CAS3, CAS9, P53*, *BIRC* and *PPIA*), RT² SYBR Green qPCR mastermix, and expression was performed using the qRT-PCR device under the conditions of hold 95˚C 15 min, cycle 95˚C 15 sec and 60˚C 30 sec, for 40 cycles. The results were recorded at 60˚C. The threshold limit was set to 0.05 and the Ct values of the samples were calculated.

The results of this article were prepared on performed *in vitro* cultured cells study. Therefore, there is no need for ethics committee approval.

Statistical analysis

Statistics of the Ct values were prepared with an internet-based program RT² Profiler PCR Data Analysis 3.5. ΔΔCt was used to interpret the gene expression data.[28] All experiments were performed twice.

RESULTS

The average Ct (mean), average ΔΔCT, and fold regulation (up-down regulation) values of the A549 cell line after exposure to pendimethalin and trifluralin for 24 h were calculated. ΔCt calculation was preferred to normalize the raw data and Ct value of the housekeeping gene, *PPIA*, was used as the normalization factor. Fold regulation is the ratio of the relative gene expression between the housekeeping gene and the treatment group. This calculation illustrated the up- and down-regulated expressions within $p<0.05$ significance. The formulas used for the calculations were as follows:

ΔCt = Ct (gene of interest) - average [Ct (housekeeping / reference gene)]

ΔΔCt = ΔCt (test group n) - ΔCt (control group)

Average ΔCt = [ΔCt (sample 1) + ΔCt (sample 2) + ... ΔCt (sample n)] / n samples

Average ΔΔCt = $2^{(-\text{Average } \Delta Ct)}$

Fold regulation = $2^{(-\Delta\Delta Ct)}$

The results are presented in Tables 2-7.

Due to the fold regulation results of trifluralin, all gene expressions were down-regulated at the examined concentrations (1-500 µM). However, pendimethalin showed different regulation profiles in the same genes. The concentrations of 100 and 500

Table 1. The PCR primers used in this study

P53	5′-CCCAGCCAAAGAAGAAACCA-3′ (fwd)
	5′-TTCCAAGGCCTCATTCAGCT-3′(rv)
BIRC5	5′-AGAACTGGCCCTTCTTGGAGG-3′(fwd)
	5′-CTTTTTATGTTCCTCTATGGGGTC-3′(rv)
BAX	5′-TGCTTCAGGGTTTCATCCAG-3′(fwd)
	5′-GGCGGCAATCATCCTCTG-3′(rv)
BCL-2	5′-AGGAAGTGAACATTTCGGTGAC-3′(fwd)
	5′-GCTCAGTTCCAGGACCAGGC-3′(rv)
CAS3	5′-ACATGGCGTGTCATAAAATACC-3′(fwd)
	5′-CACAAAGCGACTGGATGAAC-3′(rv)
CAS9	5′-CCAGAGATTCGCAAACCAGAGG-3′(fwd)
	5′-GAGCACCGACATCACCAAATCC-3′(rv)
Housekeeping ***PPIA***	5′-AAGGGTTCCTGCTTTCAC-3′(fwd)
	5′-GGACCCGTATGCTTTAGG-3′(rv)

Table 2. Average Ct values of pendimethalin

AVG Ct pendimethalin

Symbol	Control	PM 500	PM 100	PM 50	PM 10	PM 1
BAX	19.48	34.74	33.89	25.66	23.81	19.97
BCL-2	26.87	30.87	30.88	29.97	28.94	24.98
BIRC5	25.79	33.95	*N/A	31.78	29.99	22.99
P53	20.35	34.43	34.85	26.59	26.04	19.93
CAS3	22.79	*N/A	*N/A	28.57	25.94	22.05
CAS9	24.27	*N/A	33.31	30.22	25.7	22.9
PPIA	25.03	*N/A	*N/A	27.48	26.36	22.27

*N/A greater than 35

Table 3. Average ΔΔCt values of pendimethalin

$2^{(-\text{Average } \Delta Ct)}$ pendimethalin

Symbol	Control	PM 500	PM 100	PM 50	PM 10	PM 1
BAX	47.01	1.20	2.17	3.53	5.86	4.92
BCL-2	0.28	17.57	17.39	0.18	0.17	0.15
BIRC5	0.59	2.07	1.00	0.05	0.08	0.61
P53	25.72	1.49	1.11	1.86	1.24	5.06
CAS3	4.74	1.00	1.00	0.47	1.33	1.16
CAS9	1.69	1.00	3.24	0.15	1.58	0.65
PPIA	1.00	1.00	1.00	1.00	1.00	1.00

Table 4. Fold regulation (up-down) values of pendimethalin

Up-down regulation (comparing to control group) pendimethalin					
	PM 500	PM 100	PM 50	PM 10	PM 1
Symbol	Fold regulation	Fold regulation	Fold regulation	Fold regulation	Fold regulation
BAX	-39.1245*	-21.7057*	-13.3152*	-8.0278*	-9.5467*
BCL	62.6829**	62.0345**	-1.5692***	-1.6818***	-1.834***
BIRC5	3.4943**	1.6876***	-11.6318	-7.336	1.0281***
P53	-17.2677*	-23.1831*	-13.8326*	-20.6776*	-5.0806*
CAS3	-4.7404*	-4.7404*	-10.0561*	-3.5554*	-4.0699*
CAS9	-1.6935***	1.9119***	-11.2746*	-1.0718***	-2.6208*
PPIA	1	1	1	1	1

*Down regulated expression compared with the control group (*PPIA*) p<0.05
**Up regulated expression compared with the control group (*PPIA*) p<0.05
***No significant changes observed compared with the control group

Table 5. Average Ct values of trifluralin

AVG Ct trifluralin						
Symbol	Control group	TF 500	TF 100	TF 50	TF 10	TF 1
BAX	19.48	22.83	18.29	18.12	18.79	18.35
BCL	26.87	28.78	25.21	25.78	26.36	25.97
BIRC5	25.79	29.42	24.85	24.52	24.49	23.77
P53	20.35	24.86	19.87	19	19.7	19.33
CAS3	22.79	26.05	21.41	21.9	22.77	22.33
CAS9	24.27	26.12	22.55	22.65	23.21	23.16
PPIA	25.03	24.96	20.74	20.26	20.94	21.07

Table 6. Average ΔΔCt values of trifluralin

$2^{(-\text{Average }\Delta Ct)}$ trifluralin						
Symbol	Control group	TF 500	TF 100	TF 50	TF 10	TF 1
BAX	47.01	4.38	5.45	4.42	4.44	6.61
BCL-2	0.28	0.07	0.05	0.02	0.02	0.03
BIRC5	0.59	0.05	0.06	0.05	0.09	0.15
P53	25.72	1.07	1.83	2.39	2.35	3.35
CAS3	4.74	0.47	0.63	0.32	0.28	0.42
CAS9	1.69	0.45	0.28	0.19	0.21	0.24
PPIA	1.00	1.00	1.00	1.00	1.00	1.00

µg/mL, *BCL-2* gene, and on 500 µg/mL *BIRC5* gene expressions were found up-regulated compared with the *PPIA* control gene, whereas other concentrations of pendimethalin the examined genes are down-regulated.

DISCUSSION

Pesticide use has brought about both positive and negative results on human health and the environment. They led to an increase of the amount and quality of agricultural products, along with various health problems and disruption of the soil and water.

In this study, we determined the changes of apoptosis-related gene expressions with dinitroaniline herbicides. After 24 h of incubation, at the concentration of 100 µM, pendimethalin significantly down-regulated *BAX*, *P53*, and *CAS3*. Although *CAS9* levels showed no significant change, *BCL-2* and *BIRC5* levels were up-regulated with pendimethalin exposure. On the other hand, trifluralin exposure down-regulated all examined gene levels at all concentrations. It has been shown that *P53* is essential for normal cell apoptosis regulation because of its ability to control *BAX* regulation - the proapoptoic member of the *BCL-2* family.[29] Decreased *P53* levels gave rise to cellular viability, lifespan, and chromosomal instability.[30] It can be stated that increased *BCL-2* expressions come with a decrease of *P53* and *BAX* levels and prevent A549 cells from entering apoptosis. Also, *BAX* can induce caspase activation and increase cellular reactive oxygen species (ROS) by caspase cleavage.[31] Studies demonstrated that *CAS3* activation mediated the *BAX*-mediated pro-oxidant effects[32] and had an important role on inducing apoptosis via the mitochondrial cascade.[33] In our study, *CAS3* expressions were found down-regulated with *BAX*, which in turn lowered the probability of apoptosis on A549 non-small lung cancer cells.

Table 7. Fold regulation (up-down) values of trifluralin

Up-down regulation (comparing to control group) trifluralin					
	TF 500	TF 100	TF 50	TF 10	TF 1
Symbol	Fold regulation	Fold regulation	Fold regulation	Fold regulation	Fold regulation
BAX	-10.7406*	-8.6338*	-10.6295*	-10.5927*	-7.1107*
BCL-2	-3.9449*	-6.2118*	-12.8616*	-12.0003*	-8.3397*
BIRC5	-13.0412*	-10.2319*	-11.3137*	-6.9644*	-3.8504*
P53	-24.0006*	-14.0744*	-10.7406*	-10.9283*	-7.6741*
CAS3	-10.091*	-7.5685*	-14.7741*	-16.8538*	-11.3137*
CAS9	-3.7842*	-5.9587*	-8.8766*	-8.1965*	-7.1851*
PPIA	1	1	1	1	1

*Downregulated expression compared with the control group (PPIA) p<0.05
**Upregulated expression compared with the control group (PPIA) p<0.05
***No significant changes observed compared with the control group

Caspases can initiate the degradation phase of apoptosis with DNA fragmentation and blebbing.[34] *CAS9* inhibition was shown to decrease the ROS production in mitochondria,[35] and the up-regulation resulted with induced ROS production and activation of *CAS3* and *CAS7*.[36]

BIRC5 (survivin) showed different effects with herbicide exposure at the concentration of 100 µM. Although pendimethalin caused up-regulation, trifluralin exposure down-regulated *BIRC5* levels significantly. It is known that *BIRC5* is responsible for cell division regulation during the G_1-S phase and it is also considered for anticancer therapies.[37] Expressed levels of *BIRC5* were found higher than in normal healthy cells in various tumors such as lung, breast, ovarian, and prostate cancers.[38-41] One study stated that *BIRC5* silencing suppressed cell proliferation in A549 non-small lung cancer cells.[38] Compared with our results, we can state that even though pendimethalin reduced apoptotic cycles with *BIRC5* up-regulation, trifluralin exposure could not deactivate programmed cell death in A549 cells at the *BIRC5* level.

CONCLUSIONS

According to our findings and those of previous studies, pendimethalin and trifluralin exposure resulted with reduced-apoptosis, which in turn lead to tumor growth in A549 cells *in vitro*. As stated before, both herbicides significantly changed the expression levels, but pendimethalin had more effects on anti-apoptosis than trifluralin. This study found that tumor suppression genes can be altered by environmental exposure and further studies will enlighten us about the connection between dinitroaniline herbicides and lung cancer.

ACKNOWLEDGEMENTS

This work was supported by the Hacettepe BAP under Grant number 1603.

Conflict of Interest: No conflict of interest was declared by the authors.

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