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# Cellular Effects of Troglitazone on YD15 Tongue Carcinoma Cells

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**An FDA approved drug for the treatment of type II diabetes, Troglitazone (TRO), a peroxisome proliferator–activated receptor gamma agonist, is withdrawn due to severe idiosyncratic hepatotoxicity. In the search for new applications of TRO, we investigated the cellular effects of TRO on YD15 tongue carcinoma cells. TRO suppressed the growth of YD15 cells in the MTT assay. The inhibition of cell growth was accompanied by the induction of cell cycle arrest at $G_0/G_1$ and apoptosis, which are confirmed by flow cytometry and western blotting. TRO also suppressed the expression of cell cycle proteins such as cyclin D1, cdk2, cdk4, cyclin B1, cdk1(or cdc2), cyclin E1 and cyclin A. The inhibition of cell cycle proteins was coincident with the up-regulation of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$. In addition, TRO induces the activation of caspase-3 and caspase-7, as well as the cleavage of PARP. Further, TRO suppressed the expressions of Bcl-2 without affecting the expressions of Bad and Bax. Overall, our data supports that TRO induces cell cycle arrest and apoptosis on YD15 cells.**

**Key words: Cell cycle arrest, apoptosis, troglitazone, YD15 tongue carcinoma**

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## Introduction

Head and neck cancer (HNC) is the sixth common cancer worldwide [1] and one of the leading causes of death in developing countries [2]. HNC is generally diagnosed as squamous cell carcinoma (HNSCC) in oral cavity, pharynx and larynx. Chronic use of tobacco, alcohol consumption and infection by human papillomavirus (HPV) are considered as main risk factors [3-5]. Surgery, radiotherapy, chemotherapy or combinations of these are major options for the treatment of HNC. Despite the improved understanding in diagnosis and therapeutics [6,7] as well as genetic alternations [8] of HNSCC tumors, 5-year survival rate of patients is still low, at 50 % or so [9,10]. The main reason for the treatment failure is due to either the late identification of lesions at advanced stages of the disease or the recurrences and/or metastasis after treatment of the primary tumor. Thus searching new molecular target or novel drug is important strategy for HNC treatment.

Troglitazone (TRO) is a member of thiazolidinediones which was identified as a synthetic agonist for PPARγ [11]. TRO was once approved by FDA for the treatment of type II diabetes but have withdrawn from the market due to its severe idiosyncratic hepatotoxicity. Interestingly, recent reports show new aspects of TRO on cancer treatment. TRO induced cell cycle arrest at $G_1$ phase and/or apoptosis in various human cancer cells such as prostate, gastric, liver, breast, lung and cervical [14-18].

Despite the potential chemo preventive effects of TRO, few study have undergone on oral cavity cancer. In this study we have chosen YD15 tongue carcinoma cells to investigate the cellular effects of TRO. TRO suppressed cell growth and the cellular effects of TRO were associated with the cell cycle arrest at $G_0/G_1$ by inhibiting cell cycle proteins, up-regulating CDKIs (p21 and p27) as well as the apoptosis by activating caspase-3, -7 and the cleavage of PARP.

## Materials and Methods

### Chemicals and antibodies

Troglitazone was purchased from Cayman Chemical and was dissolved in dimethyl sulfoxide. Antibodies for cdk1, cdk2, cdk4, cyclin B1, cyclin A, cyclin E1, p27, FADD and caspase-3 were purchased from Santa Cruz Biotechnology Inc. (Santa cruz, CA, USA). Antibodies for p21, cleaved caspase-7 and cyclin D1 were purchased from Cell signaling Technology Inc. (Danvers, MA). PARP-p85 was purchased from Epitomics (Burlingame, CA).

### Cell culture

YD15 cells were purchased from Korean Cell Line Bank (Seoul, Korea) and were grown in RPMI-1640 media which was supplemented with 10% FBS and 1 % antibiotic antimycotic solutions. (Welgene Inc., Seoul, Korea). The cells were maintained in a humidified 5 % $CO_2$ incubator at 37 °C.

### Cell viability assay

Cell proliferation was detected by 3-(4-, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay (Sigma, USA). Cells were seeded in a 96-well plate (2 x $10^4$ cells/well). After overnight incubation, cells were treated with various concentrations of TRO in a fresh medium. After appropriate time, 200 μl of MTT working solution (0.5 mg/ml in medium) was added to each well and incubated for 4 h at 37 °C. Then, the medium was removed and 200 μl of lysis buffer (10 % sodium dodecylsulfate, 0.1 N HCl) was added to each well and incubated for additional 2 h to dissolve the formazan crystals. The UV absorbance of each sample was measured at 540 nm by using a microplate reader and determined the cell viability. All data were triplicated at a given concentration.

### DAPI staining

YD15 cells were seeded in 8-chamber slide (1 x $10^5$ cells/ml) for 24 h and then treated with TRO for 24 h. After treatment, the medium was removed and the cells were washed with PBS and fixed in 4 % paraformaldehyde for 30 min at room temperature. The fixed cells were washed with PBS and stained with DAPI (0.5 μg/ml) at room temperature in the dark for 30 minutes. The nucleus of the cell was captured by fluorescent microscopy (Zeiss, Germany).

### Cell cycle analysis by flow cytometry

Cells were seeded into a 60 mm dish (1 x $10^5$ cells/ml) for 24 h before being treated with indicated concentration of TRO (0, 50, 75, 150 μM) for 24 h. Cells were collected, fixed in 70 % ethanol, treated with RNase (200 μg/ml) and stained with propidium iodide (50 μg/ml) for flow cytometry. DNA content analyses were performed by using FACS Calibur flowcytometer (BD Biosciences). The data was analyzed with CellQuest software (BD Biosciences).

### Western blot analysis

YD15 cells were treated with indicated concentration of TRO for 24 h. Cell lysates were extracted with lysis buffer (Cell Signaling, MA) and protein concentration was determined by BCA Kit (PIERCE, IL). Protein samples (20 μg) were loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into PVDF membranes (Millipore, MA). Antibodies were used in accordance with the manufacturer's instruction. The signals were detected by enhanced chemiluminescence (ECL).

## Results

### TRO inhibited the cell growth in YD15 cells

The effect of TRO on YD15 cell growth was investigated by an MTT assay. YD15 cells were treated with various concentrations of TRO for 24 h. As shown in Figure 1, TRO significantly inhibited the cell viability of YD15 in a dose-dependent manner.

### TRO induced $G_0/G_1$ phase arrest and apoptosis in YD15 cells

To understand further on the cell growth inhibition, the effect of TRO on cell cycle was investigated by FACS

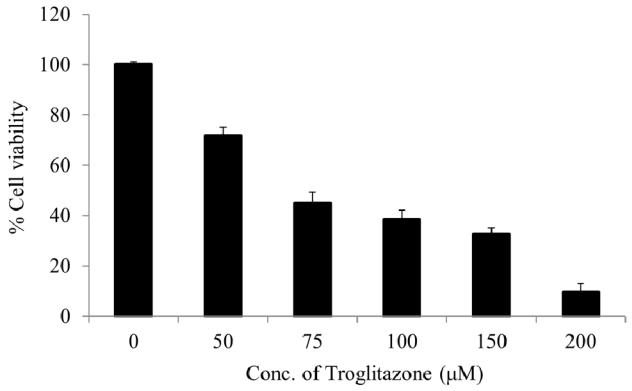


**Fig. 1.** Effect of TRO on YD15 cell viability. Cells were treated with indicated concentration of TRO for 24 h. TRO inhibited the growth of YD15 cells in a dose dependent manner. Vertical bars indicate means and standard errors (n=3).

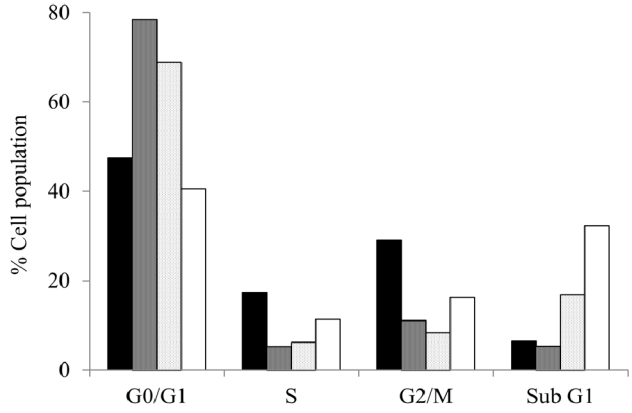


**Fig. 2.** The percent cell population in YD15 cell cycle phase. Cells were treated with indicated doses of TRO for 24 h. TRO induced cell cycle arrest at $G_0/G_1$ phase; black bar, control; dark grey, TRO 50 μM; dotted bar, TRO 75 μM; white bar, TRO 150 μM.

analysis. YD15 cells were treated with indicated concentration of TRO (0, 50, 75, 150 μM) for 24 h and analyzed by flow cytometry (Fig. 2). TRO inhibited YD15 cell growth not only by inducing cell cycle arrest but also by operating programmed cell death. The accumulation of cells in $G_0/G_1$ phase were maximized with 78.4 % in cell population at 50 μM of TRO, and then followed by 68.8 % at 75 μM. Similarly, the percent cell population in S phase and $G_2$/M phase was decreased to 5.29, 6.18 vs 17.3 of the control cells at 50 μM and 11.1, 8.37 vs 29.1 (the control) at 75 μM, respectively. TRO treatment increased sub-$G_1$ population dose-dependently. At high dose treatment (150 μ M) TRO did not exhibit the features of cell cycle arrest but increased the cell population of sub-$G_1$ with the concomitant

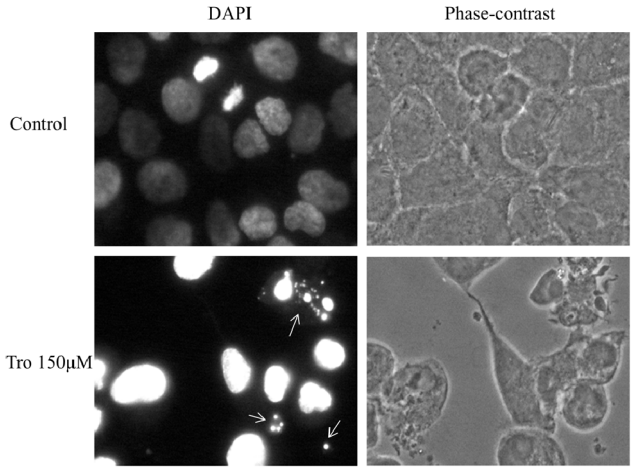


**Fig. 3.** The effects of TRO on apoptotic proteins and nucleus / cell morphology. YD15 cells were treated with an indicated concentration of TRO for 24 h, DAPI stained and phase contrasted under fluorescent microscopy. The images were captured at a magnification of x400. The arrows indicate the nuclear bodies of apoptotic cells.

decrease of cell number in cell cycle phases, probably due to apoptosis.

To convince whether the cell death induced by TRO was by apoptosis or not, we further examined the expression of apoptotic marker proteins by western blotting and also observed the image of cell nucleus by DAPI staining. DAPI staining showed fragmented morphology of nuclear bodies, confirming that TRO induced apoptosis on YD15 cells (Fig. 3). In addition, TRO treatment caused the activation of caspase-3, caspase-7 and cleavage of PARP. TRO decreased the expression of Bcl-2 without affecting on Bad and slightly decreasing Bax at 150 μM (Fig. 4).

To understand the mechanism responsible for cell cycle arrest at $G_0/G_1$ phase, the effect of TRO on cell cycle regulatory molecules (cyclins, CDKs) were examined by western blotting (Fig. 4A). TRO suppressed cyclin D1 and cdk4 which are necessary for $G_1$ phase progression, and also cdk2 which are required for $G_1$/S transition. Thereby cells were unable to go through $G_1$/S checkpoint and arrested at $G_0/G_1$ phase, which is consistent with the result obtained by FACS analysis in Figure 2. TRO also inhibited the expression of cyclin B1 and cdk1 with slight inhibition on cyclin E1 and cyclin A. Since the inhibitory action of TRO on cell growth might be involved in the expression of negative regulators of cell cycle, we examined the protein expression of CDK inhibitors, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$, by Western blotting (Fig. 4B). YD15 cells were treated with

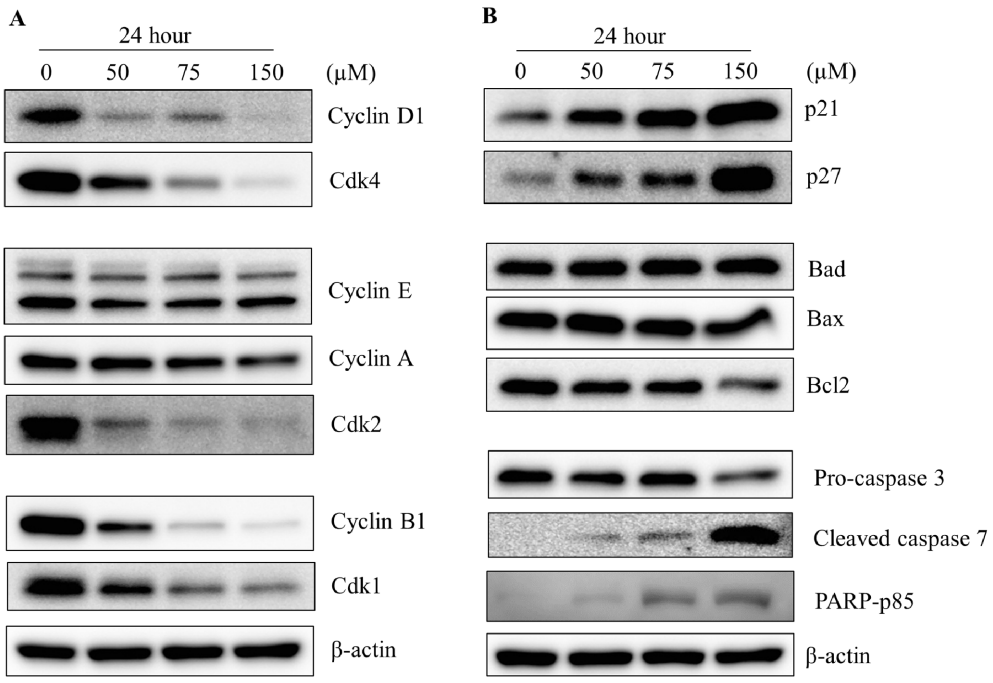


**Fig. 4.** Effects of TRO on the expression of cell cycle and apoptosis related proteins. YD15 cells were treated with indicated concentrations of TRO for 24 h; (A) the expression of cell cycle proteins and CDK inhibitors; (B) p21, p27, Bcl-2 family proteins, procaspase-3, cleaved caspase-7 and cleaved PARP (p85).

different doses of TRO for 24 h. TRO significantly increased the expression of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ proteins in a dose-dependent manner, suggesting that $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ are involved in the cell growth inhibition.

## Discussion

In this study we investigated the cellular effects of TRO on YD-15 tongue carcinoma cells. TRO inhibited the growth of YD15 cells by cell cycle arrest at $G_0/G_1$ phase at low dose condition and further induced the apoptosis at higher dose (Fig. 1 & 2). Cell cycle is tightly controlled by cyclins and cyclin-dependent kinases (CDKs). High expression of cyclin D1 has been reported in many epithelial malignancies (approximately 30 to 50 % of oral squamous carcinoma cells) [19,20]. The formation of complexes between cyclin D and cdk4 or cdk6 was required for $G_1$ phase progression [21] and the complex between cyclin E and cdk2 for the entry into S phase [22]. In our data, TRO dramatically down-regulated the expression of cyclin D1 and cdk2 (Fig. 4A), indicating that both activities of cyclin D1-cdk4 / 6 and cyclin E-cdk2 complexes were suppressed and thereby cells were arrested at $G_0/G_1$ phase without further progressing through $G_1$/S checkpoint. Consistent results were obtained by flow cytometry analysis (Fig. 2). Activities of cyclin-CDK complex are regulated by cyclin-dependent kinase inhibitors (CDKIs). $P21^{CIP1/WAF1}$ and $p27^{KIP1}$ are potent CDKIs that bind to and inhibit the activities of CDK-cyclin complex and function as a regulator of cell cycle progression [23,24]. In our results both $p27^{KIP1}$ and $p21^{CIP1/WAF1}$ were up-regulated by TRO, suggesting that the inhibition of cell cycle progression was associated with the induction of $p27^{KIP1}$ and/or $p21^{CIP1/WAF1}$ (Fig. 4B).

Since tumor-suppressor p53 is one of the major regulators of $p21^{CIP1/WAF1}$, we also examined the expression of p53 in TRO-treated YD15 cells. However, the expression level of p53 was very low (data not shown). In many tumor cells normal p53 function is lost either through mutations / genomic deletions or by up-regulation of negative regulators of p53. In YD15 cells, p53 is known mutated at codon 258 with GAA -> GCA (Glu -> Ala) [25]. Thus in our result the up-regulation of $p21^{CIP1/WAF1}$ and following cell cycle arrest by TRO treatment appears p53-independent.

The cell cycle suppression allows cells to repair damaged DNA and maintain the normal cell progression. If the

damage cannot be repaired, cells may enter to apoptosis. The activation of apoptosis is mainly mediated through the extrinsic (death receptor) and the intrinsic (mitochondrial) pathways [26]. The intrinsic apoptotic pathway is dominated by the Bcl-2 family of proteins, which governs the release of cytochrome c from the mitochondria. Some of these proteins such as Bcl-2 and Bcl-XL are anti-apoptotic while others are pro-apoptotic (Bax and Bad). The sensitivity of cells to apoptotic stimuli depends on the balance of pro- and anti-apoptotic Bcl-2 proteins. In our result, suppressed Bcl-2 as well as the significant activation of caspase-3, -7, cleaved form of PARP and nuclei fragmentation support that TRO-induced apoptosis is likely due to the intrinsic pathway. It is not clear why TRO did not effect on the expressions of Bad and Bax even under high dose condition. Previous reports indicated that the effects of TRO on Bcl-2 family proteins are cell-type dependent. TRO did not affect Bax on human liver cancer cells [15]. However, on human myeloid leukemia cells TRO up-regulated Bax while down-regulating Bcl-2 [27]. Overall our data support that TRO suppressed the growth of YD-15 tongue carcinoma cells and the cellular effects of TRO were associated with the cell cycle arrest as well as the apoptosis. Thus TRO may have a second life as an anticancer drug in tongue cancer.

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## Conflict of interest

The authors declare no conflict of interest.

## References

1. Duvvuri U, Myers JN. Cancer of the head and neck is the sixth most common cancer worldwide. Curr Probl Surg. 2009, 46, 114-117. doi: 10.1067/j.cpsurg.2008.10.002.
2. Petersen PE. Strengthening the prevention of oral cancer: the WHO perspective. Community Dent Oral Epidemiol. 2005, 33, 397-399. doi: 10.1111/j.1600-0528.2005.00251.x.
3. Hashibe M, Brennan P, Chuang SC, Boccia S, Castellsague X, Chen C, Curado M P, Dal Maso L, Daudt AW, Fabianova E, Fernandez L, Wunsch-Filho V, Franceschi S, Hayes RB, Herrero R, Kelsey K, Koifman S, La Vecchia C, Lazarus P, Levi F, Lence JJ, Mates D, Matos E, Menezes A, McClean MD, Muscat J, Eluf-Neto J, Olshan AF, Purdue M, Rudnai P, Schwartz SM, Smith E, Sturgis EM, Szeszenia-Dabrowska N, Talamini R, Wei Q, Winn DM, Shangina O, Pilarska A, Zhang ZF, Ferro G, Berthiller J, Boffetta P. Interaction between tobacco and alcohol use and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. Cancer Epidemiol Biomarkers Prev. 2009, 18, 541-550. doi: 10.1158/1055-9965.EPI-08-0347.
4. D'Souza G, Kreimer AR, Viscidi R, Pawlita M, Fakhry C, Koch WM, Westra WH, Gillison ML. Case-control study of human papillomavirus and oropharyngeal cancer. N Engl J Med. 2007, 356, 1944-1956. doi: 10.1056/NEJMoa065497.
5. Blot WJ, McLaughlin JK, Winn DM, Austin DF, Greenberg RS, Preston-Martin S, Bernstein L, Schoenberg JB, Stemhagen A, Fraumeni JF. Smoking and drinking in relation to oral and pharyngeal cancer. Cancer Res. 1988, 48, 3282-3287.
6. Remmerbach TW, Weidenbach H, Pomjanski N, Knops K, Mathes S, Hemprich A, Bocking A. Cytologic and DNA-cytometric early diagnosis of oral cancer. Anal Cell Pathol. 2001, 22, 211-221. doi: 10.1155/2001/807358.
7. Driemel O, Dahse R, Berndt A, Pistner H, Hakim SG, Zardi L, Reichert TE, Kosmehl H. High-molecular tenascin-C as an indicator of atypical cells in oral brush biopsies. Clin Oral Investig 2007, 11, 93-99. doi: 10.1007/s00784-006-0086-8.
8. Le QT, Giaccia AJ. Therapeutic exploitation of the physiological and molecular genetic alterations in head and neck cancer. Clin Cancer Res 2003, 9, 4287-4295.
9. Okamoto M, Nishimine M, Kishi M, Kirita T, Sugimura M, Nakamura M, Konishi N. Prediction of delayed neck metastasis in patients with stage I/II squamous cell carcinoma of the tongue. J Oral Pathol Med. 2002, 31, 227-233. doi: 10.1034/j.1600-0714.2002.310406.x.
10. Massano J, Regateiro FS, Januario G, Ferreira A. Oral squamous cell carcinoma: review of prognostic and predictive factors. Oral Surg Ora.l Med Oral Pathol Oral Radiol Endod. 2006, 102, 67-76. doi: 10.1016/j.tripleo.2005.07.038.
11. Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). J Biol Chem. 1995, 270, 12953-12956. doi: 10.1074/jbc.270.22.12953.
12. Kubota T, Koshizuka K, Williamson EA, Asou H, Said JW, Holden S, Miyoshi I, Koeffler HP. Ligand for peroxisome proliferator-activated receptor gamma (troglitazone) has potent antitumor effect against human prostate cancer both in vitro and in vivo. Cancer Res. 1998, 58, 3344-3352.
13. Takeuchi S, Okumura T, Motomura W, Nagamine M, Takahashi N, Kohgo Y. Troglitazone induces G1 arrest by p27(Kip1) induction that is mediated by inhibition of proteasome in human gastric cancer cells. Jpn J Cancer Res. 2002, 93, 774-782. doi: 10.1111/j.1349-7006.2002.tb01319.x.
14. Motomura W, Takahashi N, Nagamine M, Sawamukai M,

Tanno S, Kohgo Y, Okumura T. Growth arrest by troglitazone is mediated by p27Kip1 accumulation, which results from dual inhibition of proteasome activity and Skp2 expression in human hepatocellular carcinoma cells. Int J Cancer. 2004, 108, 41-46. doi: 10.1002/ijc.11561.

15. Zhou YM, Wen YH, Kang XY, Qian HH, Yang JM and Yin ZF. Troglitazone, a peroxisome proliferator-activated receptor gamma ligand, induces growth inhibition and apoptosis of HepG2 human liver cancer cells. World J Gastroenterol. 2008, 14, 2168-2173. doi: 10.3748/wjg.14.2168.
16. Yin F, Wakino S, Liu Z, Kim S, Hsueh W A, Collins A R, Van Herle A J and Law R E. Troglitazone inhibits growth of MCF-7 breast carcinoma cells by targeting G1 cell cycle regulators. Biochem Biophys Res Commun. 2001, 286, 916-922. DOI:10.1006/bbrc.2001.5491
17. Li M, Lee TW, Yim AP, Mok TS, Chen GG. Apoptosis induced by troglitazone is both peroxisome proliferator-activated receptor-gamma- and ERK-dependent in human non-small lung cancer cells. J Cell Physiol. 2006, 209, 428-438. doi: 10.1002/jcp.20738.
18. Ye J, Yin L, Xie P, Wu J, Huang J, Zhou G, Xu H, Lu E, He X. Antiproliferative effects and molecular mechanisms of troglitazone in human cervical cancer in vitro. Onco Targets Ther 2015, 8, 1211-1218. doi: 10.2147/OTT.S79899.
19. Rousseau A, Lim MS, Lin Z, Jordan RC. Frequent cyclin D1 gene amplification and protein overexpression in oral epithelial dysplasias. Oral Oncol. 2001, 37, 268-275. doi: 10.1016/S1368-8375(00)00114-7.
20. Nimeus E, Baldetorp B, Bendahl PO, Rennstam K, Wennerberg J, Akervall J and Ferno M. Amplification of the cyclin D1 gene is associated with tumour subsite, DNA non-diploidy and high S-phase fraction in squamous cell carcinoma of the head and neck. Oral Oncol. 2004, 40, 624-629. doi: 10.1016/j.oraloncology.2003.12.014.
21. Donjerkovic D, Scott DW. Regulation of the G1 phase of the mammalian cell cycle. Cell Res. 2000, 10, 1-16. doi: 10.1038/sj.cr.7290031.
22. Dulic V, Lees E, Reed SI. Association of human cyclin E with a periodic G1-S phase protein kinase. Science. 1992, 257, 1958-1961. doi: 10.1126/science.1329201.
23. Gartel AL, Radhakrishnan SK. Lost in transcription: p21 repression, mechanisms, and consequences. Cancer Res. 2005, 65, 3980-3985. doi: 10.1158/0008-5472.CAN-04-3995.
24. Koga H, Sakisaka S, Harada M, Takagi T, Hanada S, Taniguchi E, Kawaguchi T, Sasatomi K, Kimura R, Hashimoto O, Ueno T, Yano H, Kojiro M, Sata M. Involvement of p21 (WAF1/Cip1), p27(Kip1), and p18(INK4c) in troglitazone-induced cell-cycle arrest in human hepatoma cell lines. Hepatology. 2001, 33, 1087-1097. doi:10.1053/jhep.2001.24024.
25. Lee EJ, Kim J, Lee SA, Kim EJ, Chun YC, Ryu MH, Yook JI. Characterization of newly established oral cancer cell lines derived from six squamous cell carcinoma and two mucoepidermoid carcinoma cells. Exp Mol Med. 2005, 37, 379-390. doi:10.1038/emm.2005.48
26. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007, 35, 495-516. doi: 10.1080/01926230701320337.
27. Liu JJ, Huang RW, Lin DJ, Peng J, Wu XY, Lin Q, Pan XL, Song YQ, Zhang MH, Hou, Chen F. Expression of survivin and bax/bcl-2 in peroxisome proliferator activated receptor-gamma ligands induces apoptosis on human myeloid leukemia cells in vitro. Ann Oncol. 2005, 16, 455-459. doi: 10.1093/annonc/mdi077.