Pharmaceutical Sciences, 2020, 26(2), 159-164
doi:10.34172/PS.2020.3
https://ps.tbzmed.ac.ir/

Research Article

The Sensitization of Melatonin in Osteosarcoma Cells by Suppression of Anti-Apoptotic Proteins

Seyed Mostafa Mir[1,2,3], Bahman Yousefi[4]*, Abdoljalal Marjani[5], Mahdi Rahimi[4], Durdi Qujeq[1,2,3]*

[1]*Cellular and Molecular Biology Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran.*
[2]*Department of Clinical Biochemistry, Babol University of Medical Sciences, Babol, Iran.*
[3]*Student Research Committee, Babol University of Medical Sciences, Babol, Iran.*
[4]*Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.*
[5]*Metabolic Disorders Research Center, Department of Biochemistry and Biophysics, Gorgan Faculty of Medicine, Golestan University of Medical Sciences, Gorgan, Iran.*

Article Info

Article History:
Received: 2 September 2019
Accepted: 6 December 2019
ePublished: 27 June 2020

Keywords:
-Melatonin
-Doxorubicin
-Osteosarcoma
-Combination therapy

Abstract

Background: Investigation of anti-cancer agents with desirable selective toxicity is critical for cancer therapy. The use of natural adjuvants can be a promising option in reducing the toxicity of the anti-cancer agent. The aim of this study was to investigate the potential application of melatonin (MLT) as a natural adjuvant molecule along with doxorubicin (DOX) to induce cytotoxicity in osteosarcoma (OS) cells.

Methods: Human OS cell lines included Saos-2, MG-63, and Human Bone Marrow Mesenchymal Stem Cells (hBM-MSCs) were treated with free DOX, free MLT, DOX-loaded NPs (DOX-NPs), MLT-loaded NPs (MLT-NPs), combination of DOX and MLT (DOX-MLT) and combination of DOX and MLT-loaded NPs (DOX-MLT-NPs) in separated cell culture. Cell proliferation of experiments were evaluated by MTT assay after 24 h. Total protein levels were determined by enzyme immunoassay ELISA.

Results: Herein, we found the combination of MLT with DOX, especially formulated in nano-form, is resulted in a significant reduction in the protein levels of both X-linked Inhibitor of Apoptosis (XIAP) and Survivin ($p<0.0001$). Indeed, there was a significant decrease in the expression of XIAP and Survivin when MLT is combined with DOX compared to the individual treatments.

Conclusion: Our findings indicated the synergism of the antitumor effect could be due to the down-regulation of XIAP and Survivin in the levels of protein.

Introduction

Osteosarcoma (OS) is the most frequent primary bone malignancy with the highest incidence, usually occurs in the second and third decades of life.[1] Patients are generally treated with chemotherapeutic agents following surgery.[2] However, most of the patients with OS indicate a poor prognosis to conventional chemotherapeutic agents such as doxorubicin (DOX) which can be due to metastasis and intrinsic or acquired chemotherapeutic resistance.[3] Therefore, new therapeutic strategies are critically needed for OS patients.[4] Most of the current studies in this field have been focused on the combination therapy with naturally compounds, which reduces the effective dose and consequently adverse side effects.[5,6]

Different mechanisms elucidate the drug resistance and dysregulation of apoptosis, which are strongly associated with therapeutic failure.[7-9] Inhibitor of apoptosis proteins (IAPs) area group of negative regulators of caspases and cell death.[10,11]

Overexpression of IAPs, such as X-linked Inhibitor of Apoptosis (XIAP) and Survivin in many tumors, are directly related to resistance against the apoptosis and subsequently, chemotherapeutics agents.[12-15] Therefore, identifying the potential sensitizers, which are capable of inhibiting apoptosis resistance by targeting XIAP and Survivin as well as therapy via nanocarriers, are essential in cancer treatment.

The use of melatonin (MLT), as an adjuvant and target therapy by nanocarriers, obtains much more attention for the cancer treatment.[16,17] MLT has shown a wide range of biological functions, including pro-oxidant activity in the cancer cells, anti-oxidant functions in normal cells, oncostatic activities through antiproliferative and pro-apoptotic capability an also immunomodulatory properties.[18-20] In the present study, we evaluated the effect

*Corresponding Authors: Durdi Qujeq, E-mail: dqujeq@gmail.com & Bahman Yousefi, E-mail: bahmanusefi@gmail.com

of MLT along with DOX in both forms of free and loading on nanocarriers as well as apoptosis resistance through targeting Survivin and XIAP in OS cell lines.

Materials and Methods

Materials

Cell culture materials included RPMI-1640 medium, Fetal Bovine Serum (FBS), and trypsin/EDTA solution were provided from Gibco® (Invitrogen, USA). Penicillin/streptomycin was obtained from Biowest (Nuaillè, France). Chemical material and reagents were included MLT (Sigma-Aldrich, USA), DOX (Sigma-Aldrich, USA). Human Survivin ELISA Kit and XIAP ELISA Kit were purchased from Bioassay Technology laboratory Co. Thiazolyl blue tetrazolium bromide (MTT) was provided from Bio Basic Co.

Cell culture

For cell culture experiments, Human OS cell line Saos-2, Human OS cell line MG-63, and Human Bone Marrow Mesenchymal Stem Cells(hBM-MSCs) were provided from the Pasteur Institute of Iran (Tehran, Iran). Saos-2, MG-63, and hBM-MSCs were commonly cultured in RPMI-1640 medium containing 10% FBS and 1% Penicillin-Streptomycin and were incubated at 37 °C, 5% CO_2atmosphere, as well as 95% humidity. The cells were passaged every two to three days to preserve exponential growth.

Cell proliferation assay

Determination of cell proliferation was evaluated via the MTT assay. The experiment was divided into eight groups, including (1) control group without any treatment, (2) NPs group (3) free DOX group, (4) free MLT group, (5) DOX-loaded NPs (DOX-NPs) group , (6) MLT-loaded NPs (MLT-NPs) group, (7) combination of DOX and MLT (DOX-MLT) group, and (8) combination of DOX and MLT-loaded NPs (DOX-MLT-NPs) group. Briefly, Saos-2, MG-63, and hBM-MSCs were seeded into 96-well plates (10×10^3 cells per well) and incubated in 37 °C incubator equipped with 5% CO_2 atmosphere and 95% humidity for 24 hours to attach and grow at the bottom of each well.

Then, a fresh culture growth medium,containing different drugs concentration for 24 h, was replaced. After 24 h, the media were removed and wells were washed twice with Phosphate Buffered Saline (PBS) , then the MTT solution (5 mg mL^{-1}) was added to each well and was incubated for 4 h. MTT solution was cautiously removed and 200 µL of DMSO was added to each well to dissolve formed blue formazan crystals.[21] The absorbance of solubilized formazan was measured by an enzyme-linked immunosorbent assay (ELISA) reader (Awareness Technology, Palm City, FL, USA) at 570 nm along with a reference wavelength of 630 nm. All experiments were done in triplicate, and the cell viability percentages were obtained using the following equation:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance}_{(\text{test})}}{\text{Absorbance}_{(\text{control})}} \times 100 \quad \text{Eq. (1)}$$

Measurement of Survivin and XIAP proteins

Determination of the levels of Survivin and XIAP proteins were performed by enzyme immunoassay ELISA kit specific. Briefly, Saos-2, MG-63, and hBM-MSCs were seeded into 6-well plates (5×10^5 cells per well) and incubated in 37 °C incubator, equipped with 5% CO_2 atmosphere and 95% humidity for 24 hours to attach and grow at the bottom of each well. Then, a fresh culture growth medium, containing different drugs concentration for 24 h, was replaced. After 24 h, the media were removed, and wells were washed twice with PBS solution, and then the level of Survivin and XIAP was determined by ELISA method. All experiments were done in duplicate.

Statistical analysis

Results were reported as mean ± SD from at least three independent experiments. Statistical analysis was done by using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA). One-way analysis of variance (ANOVA) was performed to determine the significance of differences among study groups. Significant differences were shown as *p<0.05, **p<0.01, ***p<0.001 and ***p<0.0001.

Result and Discussion

Cell proliferation assay

MTT assay is an essential item for evaluation of cytotoxic effects of samples (such as drug and drug carrier). In this regard, different cell lines (i.e., Saos-2, MG-63, and hBM-MSC) were treated with samples, including free DOX, free MLT, DOX-NPs, and MLT-NPs. Additionally, the combination forms of the above samples (DOX-MLT and DOX-MLT-NPs) were used to show the synergistic effects of MLT within DOX chemotherapeutic agents on cancer cells and the result has been illustrated in Figure 1.

First, the Saos-2 cells exposed to above-mentioned samples, and the results showed that DOX, DOX-NPs, DOX-MLT, and DOX-MLT-NPs demonstrated dose-dependent cell proliferation inhibition. Interestingly, MLT has no cytotoxic effects on Saos-2 cells, and just has slight changes in cell viability. However, MLT-NPs illustrated dose-dependent effects on cell proliferation inhibition, which may be attributed to the MT1 and MT2 on the cell membrane.

Due to the internalization of MLT-NPs via endocytosis mechanism, MLT-NPs do not depend on the MLT receptors for entrance, and could quickly affect the cells. Whereas, free MLT was completely controlled by MT1 and MT2 receptors for internalizations.[22] Although the cell suppression capacity of MLT was weaker than DOX, it is promising that the cytotoxicity of free DOX/MLT and DOX-MLT-NPs was higher than DOX.

According to the above-mentioned results, MG-63 cells were also showed similar outcomes; but with this difference that all the treated samples showed weaker effects with a

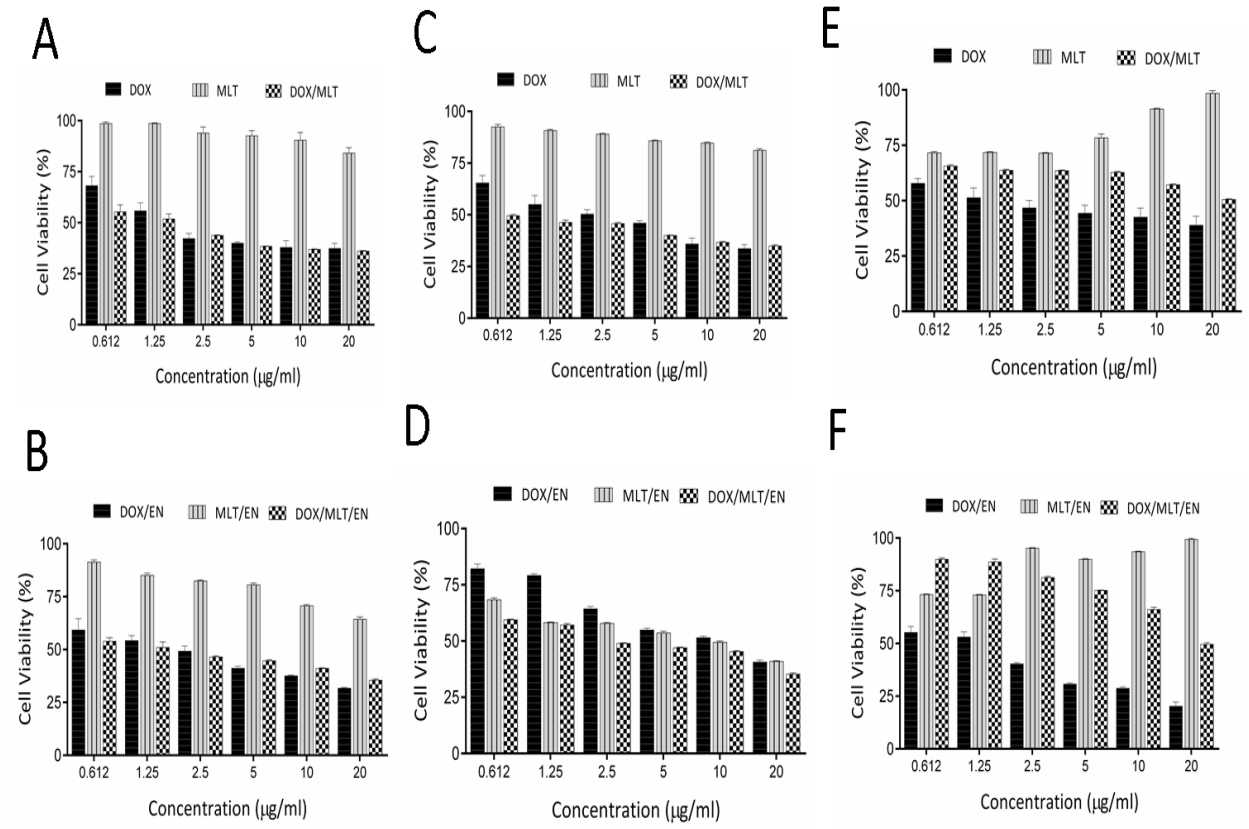

Figure 1. Cytotoxicity of free DOX, DOX-loaded nanocarrier, free MLT, MLT loaded nanocarrier, free DOX/MLT, DOX/MLT loaded nanocarrier against Saos-2 (A and B), MG-63 (C and D), and hBM-MSC (E and F).

dose-dependent pattern for cell proliferation inhibition in MG-63 cell line. However, it was observed that in the MG-63 results there is a significant effect of the MLT on the viability of the cells in comparison to the Saos-2, which has significantly reduced the number of cells. Similar to the results of Saos-2, MLT enhanced the toxic effects of DOX in both treated DOX-MLT and DOX-MLT-NPs groups. The hBM-MSC line was chosen to show the cytoprotective effects of MLT on normal cells.

XIAP and Survivin protein levels on Saos-2, MG-63,and hBM-MSC lines

The expression of XIAP and Survivin were measured at the levels of proteins in Saos-2, MG-63,and hBM-MSC lines. MLT and DOX were used alone and in combination. All three cell lines, treated with MLT and DOX at IC_{50} concentration, were showed a significant reduction in the protein expression levels of both XIAP and Survivin (Figure 2 and 3). Indeed, a combination of MLT and DOX significantly decreased XIAP and Survivin at the levels of proteins when compared with MLT or DOX mono treatment. On the other hand, the treatment of DOX and MLT loaded on NPs statistically showed a significant decreased in the protein levels of XIAP and Survivin. Accumulating recent studies have been focused on the IAPs because of their critical role in oncogenesis.[23,24]

The down-regulation of XIAP and Survivin proteins in hBM-MSC lines may be related to the other effects of MLT. As mentioned in several studies, MLT has a wide effect in biological processes including the regulation of mitochondrial activities in the non-tumor cells, pro-oxidant activity in cancer cell and antioxidant activity in normal cells especially in hBM-MSC lines.[25,26]

Different studies have demonstrated a significant correlation between IAPs expression levels and poor prognosis in malignant diseases.[27-29] It was demonstrated that down-regulation of IAPs l increases the sensitivity of cancer cells to chemotherapeutics agents.[13,30,31] The main findings of this study are in agreement with other researchers findings. In this regards, Lulu Fan et al. proposed that MLT synergistically induced cancer cells to apoptosis through targeting Survivin and XIAP.[32] In another study, Lulu Fan et al. also demonstrated that MLT overcame apoptosis resistance in human hepatocellular carcinoma by targeting Survivin and XIAP.[33] MLT significantly inhibited the growth of HepG2 and SMMC-7721 cells and stimulated apoptosis through down-regulation of Survivin and XIAP.[33] Our results indicated that MLT, along with DOX, significantly exerts more potent effects through reduction of XIAP and Survivin protein levels in Saos-2, MG-63, and hBM-MSC lines. The expression pattern of free drugs and encapsulation form are summarized in Figure 2 and 3 respectively.

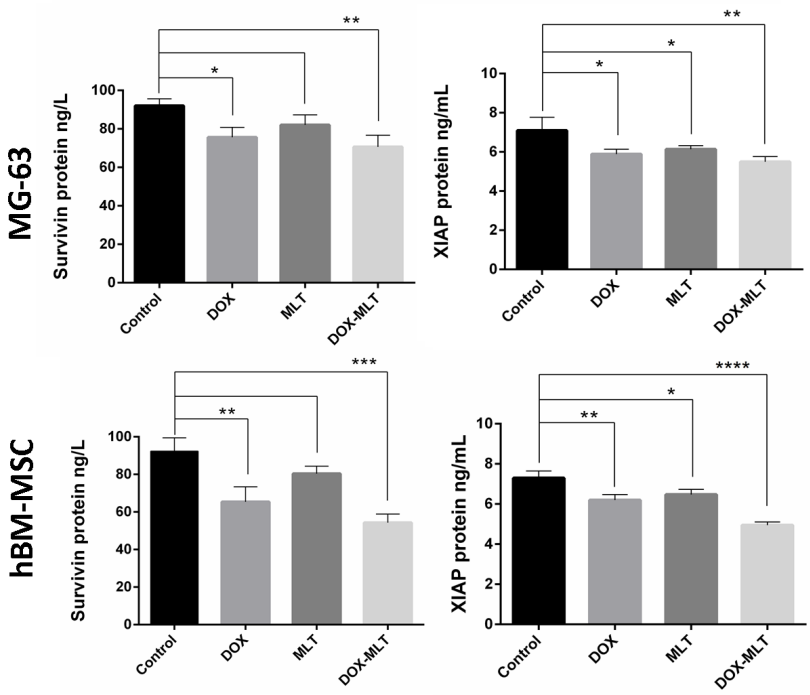

Figure 2. The effects of free MLT and free DOX on the levels of XIAP and Survivin proteins in the Saos-2, MG-63, and hBM-MSC lines. doxorubicin (DOX), melatonin (MLT).

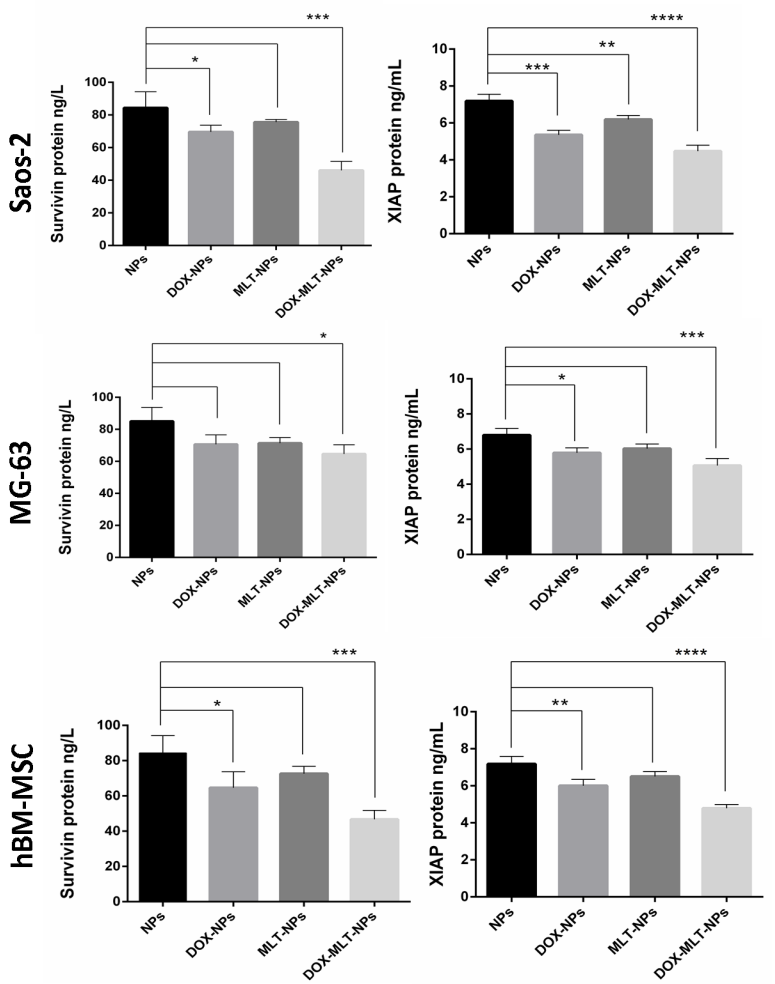

Figure 3. The effects of MLT-NPs and DOX-NPs on the levels of ꞵXIAP and Survivin proteins in the Saos-2, MG-63, and hBM-MSC lines. Nanoparticles (NPs), doxorubicin (DOX), melatonin (MLT).

Conclusion

In conclusion, these results indicate that MLT, in combination with DOX, has significantly synergistic effects on the human OS cell lines Saos-2 and MG-63, which may be related to down-regulation of Survivin and XIAP. MLT is expected to be an adjuvant drug in OS treatment. Indeed, MLT facilitates the efficacy of DOX in the stimulation of the processes, which leads to cell death. Loading MLT in NPs enhances the effectiveness of DOX in cell proliferation in OS cell lines. We suggest that the application of MLT-NPs, accompanied by chemotherapeutic agents, can be considered as a promising agent to increase the efficacy of chemotherapeutics for the treatment of OS patients.

Conflict of Interests

The authors declare that they had no conflict of interests.

References

1. Misaghi A, Goldin A, Awad M, Kulidjian AA. Osteosarcoma: A comprehensive review. SICOT J. 2018;4:12. doi:10.1051/sicotj/2017028
2. Ottaviani G, Jaffe N. The epidemiology of osteosarcoma. Cancer Treat Res. 2009;152:3-13. doi:10.1007/978-1-4419-0284-9_1
3. Lindsey BA, Markel JE, Kleinerman ES. Osteosarcoma overview. Rheumatol Ther. 2017;4(1):25-43. doi:10.1007/s40744-016-0050-2
4. Sayles LC, Koehne A, Marini K, Lee AG, Leung SG, Shah AT, et al. Targeted drug therapies for osteosarcoma. In: Proceedings: AACR Annual Meeting 2019; March 29-April 3, 2019; Atlanta, GA: AACR; Cancer Res 2019;79(13 Suppl):Abstract nr 2880.
5. Lohse I, Wildermuth E, Brothers SP. Naturally occurring compounds as pancreatic cancer therapeutics. Oncotarget. 2018;9(83):35448. doi:10.18632/oncotarget.26234
6. Zhang Y, Yang J, Zhao N, Wang C, Kamar S, Zhou Y, et al. Progress in the chemotherapeutic treatment of osteosarcoma. Oncol Lett. 2018;16(5):6228-37. doi:10.3892/ol.2018.9434
7. Mir SM, Samadian E, Sadeghi SH, Khoshbin Khoshnazar A. In vivo analysis of h2ax phosphorylation induced by γ-radiation. Med Lab J. 2017;11(2):11-5. doi:10.18869/acadpub.mlj.11.2.11
8. Mir SM, Samadian E, Alijanpour S, Khoshbin Khoshnazar A, Haghighatfard H, Sadeghi SH. Impact of ionizing radiation on the expression of cdc25a phosphatase (in vivo). Med Lab J. 2016;10(5):22-6. doi:10.18869/acadpub.mlj.10.5.22
9. Yousefi T, Mir SM, Asadi J, Tourani M, Karimian A, Maniati M, et al. In silico analysis of non-synonymous single nucleotide polymorphism in a human klk-2 gene associated with prostate cancer. Meta Gene. 2019;21:100578. doi:10.1016/j.mgene.2019.100578
10. Finlay D, Teriete P, Vamos M, Cosford ND, Vuori K. Inducing death in tumor cells: Roles of the inhibitor of apoptosis proteins. F1000Res. 2017;6:587. doi:10.12688/f1000research.10625.1
11. Storm MB, Junker M. Dissecting the function of iap (inhibitor of apoptosis) protein domains in inhibiting an apoptotic caspase. FASEB J. 2018;32(1_supplement):528.15.
12. Yang WZ, Zhou H, Yan Y. Xiap underlies apoptosis resistance of renal cell carcinoma cells. Mol Med Rep. 2018;17(1):125-30. doi:10.3892/mmr.2017.7925
13. Rathore R, McCallum JE, Varghese E, Florea A-M, Büsselberg D. Overcoming chemotherapy drug resistance by targeting inhibitors of apoptosis proteins (IAPs). Apoptosis. 2017;22(7):898-919. doi:10.1007/s10495-017-1375-1
14. Li J, Han Y, Zhou D, Zhou Y, Ye M, Wang H, et al. Downregulation of survivin gene expression affects ionizing radiation resistance of human t98 glioma cells. Cell Mol Neurobiol. 2018;38(4):861-8. doi:10.1007/s10571-017-0560-7
15. Hehlgans S, Petraki C, Reichert S, Cordes N, Rödel C, Rödel F. Double targeting of survivin and xiap radiosensitizes 3d grown human colorectal tumor cells and decreases migration. Radiother Oncol. 2013;108(1):32-9. doi:10.1016/j.radonc.2013.06.006
16. Farhood B, Goradel NH, Mortezaee K, Khanlarkhani N, Salehi E, Nashtaei M, et al. Melatonin as an adjuvant in radiotherapy for radioprotection and radiosensitization. Clin Transl Oncol. 2019;21(3):268-79. doi:10.1007/s12094-018-1934-0
17. Najafi M, Salehi E, Farhood B, Nashtaei MS, Hashemi Goradel N, Khanlarkhani N, et al. Adjuvant chemotherapy with melatonin for targeting human cancers: A review. J Cell Physiol. 2019;234(3):2356-72. doi:10.1002/jcp.27259
18. Reiter R, Rosales-Corral S, Tan DX, Acuna-Castroviejo D, Qin L, Yang SF, et al. Melatonin, a full service anti-cancer agent: Inhibition of initiation, progression and metastasis. Int J Mol Sci. 2017;18(4):843. doi:10.3390/ijms18040843
19. Talib W. Melatonin and cancer hallmarks. Molecules. 2018;23(3):518. doi:10.3390/molecules23030518
20. de Almeida Chuffa LG, Seiva FRF, Cucielo MS, Silveira HS, Reiter RJ, Lupi LA. Clock genes and the role of melatonin in cancer cells: An overview. Melatonin Res. 2019;2(2):133-57. doi:10.32794/mr11250026
21. Yarmohamadi A, Asadi J, Gharaei R, Mir M, Khoshnazar AK. Valproic acid, a histone deacetylase inhibitor, enhances radiosensitivity in breast cancer cell line. J Radiat Cancer Res. 2018;9(2):86. doi:10.4103/jrcr.jrcr_37_17
22. Legros C, Devavry S, Caignard S, Tessier C, Delagrange P, Ouvry C, et al. Melatonin mt1 and mt2 receptors display different molecular pharmacologies only in the g-protein coupled state. Br J Pharmacol. 2014;171(1):186-201. doi:10.1111/bph.12457
23. Ndubaku C, Cohen F, Varfolomeev E, Vucic D. Targeting inhibitor of apoptosis proteins for therapeutic intervention. Future Med Chem. 2009;1(8):1509-25.

doi:10.4155/fmc.09.116
24. Bouaouiche S, Dubrez L, Bettaieb A, Plenchette S. Iaps: Mediators of oncogenesis and targets for anticancer therapy. Crit Rev Oncog. 2016;21(5-6):399-411. doi:10.1615/critrevoncog.2017021084
25. Reiter R, Rosales-Corral SA, Tan DX, Acuna-Castroviejo D, Qin L, Yang SF, et al. Melatonin, a full service anti-cancer agent: Inhibition of initiation, progression and metastasis. Int J Mol Sci. 2017;18(4):843. doi:10.3390/ijms18040843
26. Reiter RJ, Tan DX, Rosales-Corral S, Galano A, Zhou XJ, Xu B. Mitochondria: Central organelles for melatonin's antioxidant and anti-aging actions. Molecules. 2018;23(2):509. doi:10.3390/molecules23020509
27. Chen L, Liang L, Yan X, Liu N, Gong L, Pan S, et al. Survivin status affects prognosis and chemosensitivity in epithelial ovarian cancer. Int J Gynecol Cancer. 2013;23(2):256-63. doi:10.1097/igc.0b013e31827ad2b8
28. Rosato A, Menin C, Boldrin D, Dalla Santa S, Bonaldi L, Scaini MC, et al. Survivin expression impacts prognostically on nsclc but not sclc. Lung Cancer. 2013;79(2):180-6. doi:10.1016/j.lungcan.2012.11.004
29. Gao X, Zhang L, Wei Y, Yang Y, Li J, Wu H, et al. Prognostic value of xiap level in patients with various cancers: A systematic review and meta-analysis. J Cancer. 2019;10(6):1528-37. doi:10.7150/jca.28229
30. Saralamma VVG, Lee HJ, Raha S, Lee WS, Kim EH, Lee SJ, et al. Inhibition of iap's and activation of p53 leads to caspase-dependent apoptosis in gastric cancer cells treated with scutellarein. Oncotarget. 2018;9(5):5993. doi:10.18632/oncotarget.23202
31. Silke J, Vince J. Iaps and cell death. Apoptotic and non-apoptotic cell death: Cham: Springer International Publishing ; 2016. p. 95-117.
32. Fan LL, Sun GP, Wei W, Wang ZG, Ge L, Fu WZ, et al. Melatonin and doxorubicin synergistically induce cell apoptosis in human hepatoma cell lines. World J Gastroenterol. 2010;16(12):1473-81. doi:10.3748/wjg.v16.i12.1473
33. Fan L, Sun G, Ma T, Zhong F, Wei W. Melatonin overcomes apoptosis resistance in human hepatocellular carcinoma by targeting s urvivin and xiap. J Pineal Res. 2013;55(2):174-83. doi:10.1111/jpi.12060