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The effects of melatonin and its analogues on steroidogenesis pathway: a possible therapeutical or adverse effect

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ABSTRACT: Melatonin (MLT) is a hormone that is secreted from pineal gland and regulates the circadian rhythm. It is reported to have anticancer effects in hormone dependent breast cancer via endocrine modulation, namely by antagonising the estrogen receptor (ER) or by inhibiting aromatase enzyme. However, its use is restricted because of its short half-life and poor bioavailability. Therefore, indole derived MLT analogues were synthesized previously, and they were shown to have potential antioxidant and anticancer effects. The present research is aimed to investigate the potential of MLT and its two newly synthesized analogues (M6 and M20) on steroidogenesis pathway by using an OECD validated in vitro method, H295R steroidogenesis assay (TG-456 test guideline). The compounds were incubated with H295R, human adenocarcinoma cells, for 48 hours and the hormone levels (testosterone; T and estradiol; E2) were detected by LC-MS/MS. The partial validation of the method was performed by using reference compounds forskolin, prochloraz, letrozole and ketoconazole. MLT decreased both E2 and T levels and its effect on E2 levels were dose dependent. On the other hand, M6 and M20 showed biphasic effects on both hormone levels. None of the compounds decreased H295R cell viability. These results demonstrate that depending on the use and the dose of these melatonin analogues, their potential effects on hormone production can result either in a therapeutical effect (such as anticancer agent) or a non-targeted endocrine related adverse effect when used as a pharmaceutical.

KEYWORDS: Steroidogenesis; melatonin analogues; therapeutical effect; endocrine related adverse effect; LC-MS/MS.

1. INTRODUCTION

Melatonin (MLT) is a hormone that carries an indole ring and is majorly secreted from the pineal gland. Besides, MLT synthesis also takes place in the retina, thymus, bone marrow, skin, and gastrointestinal tract. The main role of MLT is the regulation of circadian rhythm physiologically [1]. The use of MLT in the treatment of some pathological conditions such as sleep disorders, jetlag, depression, diabetes, some hormone-dependent cancers and neurodegenerative diseases is reported to be beneficial [2]. Moreover, it is reported to have anti-cancer effect in various tumor types, especially estrogen receptor positive (ER +) breast cancer [3,4] through various mechanisms [5-7]. Its anticancer effect in estrogen dependent breast cancer was suggested to result from either its ER antagonist [8] or aromatase inhibitory effect [9,10]. As a result of both effects estrogen synthesis is inhibited and/or estrogen triggered physiological processes such as proliferation of ER (+) breast cancer cells are decreased. However, there are some disadvantages in using this molecule as a drug; its short half-life, rapid metabolic inactivation, and poor bioavailability [11]. Hereby, new indole derived MLT analogues were synthesized by our group and by others [12,13]. In our previous studies our group has synthesized and evaluated antioxidant and chemopreventive effects of some novel MLT analogues [14,15]. Among many others, M6, 2-methyl-1-H-indole-3-carboxyaldehyde(4-chlorophenyl) hydrazone, was found to inhibit aromatase potently with IC50 of 8.6×10^{-6} [13].

Aromatase is the key enzyme located in the last step of steroidogenesis pathway and it catalyses the biosynthesis of estrogens from androgenic steroid hormones. Beside aromatase, many enzymes located in

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previous steps of steroidogenesis can also be therapeutical targets in the treatment of ER (+) breast cancer. Steroidogenesis pathway starts with the cleavage of side chain of the cholesterol by CYP11A and continues with conversion of pregnenolone to progesterone [16]. With further reactions catalysed by CYP17, 3β-HSD, 17β-HSD and CYP19A (aromatase), steroid hormones including testosterone, estrone and 17β-estradiol are synthesized [17]. Besides being a therapeutical target in treatment of hormone-dependent breast cancer, enzymes involved in steroidogenesis pathway can also be targets in occurrence of endocrine related adverse/toxic effects of pharmaceuticals [18,19] /endocrine disrupting chemicals (EDCs) [20,21]. To determine the potential EDCs, Organisation for Economic Co-operation and Development (OECD) has developed 5-stepped test strategy that include in silico approaches and standardized in vitro and in vivo test methods [22]. H295R steroidogenesis assay is among the commonly preferred high-throughput in vitro screening test methods that can reveal a possible mechanism of action of EDCs which may lead to adverse outcomes via disrupting steroid hormone production. H295R is a human adrenocarcinoma cell line that expresses all key enzymes and produces all steroid hormones involved in steroidogenesis. Thus, this cell line is the most widely used in vitro cellular system to study endocrine-related effects of chemicals on steroid hormone production, steroidogenic enzymes, and related gene expression [23].

Our previous studies showed that MLT and its analogues can inhibit aromatase enzyme involved in the last step of the steroidogenesis. Considering that these compounds may also affect the enzymes at first steps of the pathway, the present study is aimed to investigate potential inducer or inhibitory effects of MLT and its selected analogues (M6 and M20) (Figure 1) on the entire steroidogenesis pathway [42]. M6 (2-methyl-1-H-indole-3-carboxyaldehyde(4-chlorophenyl) hydrazone) was selected as a potent aromatase inhibitor from our previous studies [13], where its dechlorinated analogues M20 (2-methylindole-3-carboxyaldehyde phenyl hydrazone) is selected to investigate the effect of halogenation on steroidogenic enzymes.

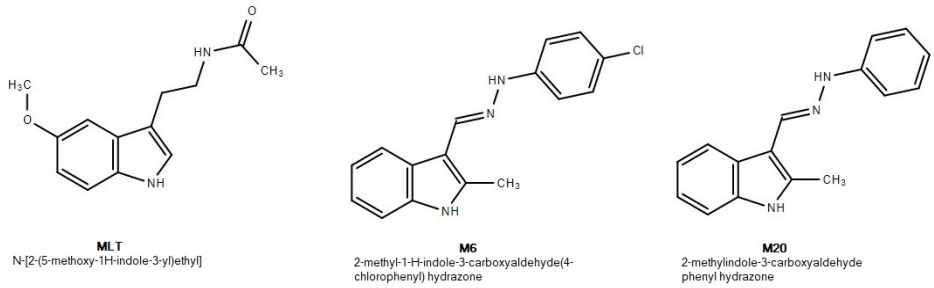

Figure 1. Chemical structures of MLT and its analogues M6 and M20 [42].

2. RESULTS

2.1. Method validation

2.1.1. H295R steroidogenesis assay

Prior to the analysis of the reference and test compounds, the proficiency test of the H295R cell line was applied according to the OECD TG-456 test guideline [17]. Data are shown in Table 1. It is demonstrated that the method worked successfully according to the performance criteria of the OECD TG-456 test guideline.

Table 1. Partial validation of H295R steroidogenesis assay.

		OECD TG-456 QC[a] plate	Our Laboratory QC plate
Estradiol (E2)	**Basal hormone level in SC[b]**	≥ 2.5 times the LOQ[c]	≥ 46.2 times the LOQ
	Induction (10 µM Forskolin)	≥ 7.5 times the SC	≥ 11.6 times the SC
	Inhibition (1 µM prochloraz)	≤ 0.5 times the SC	≤ 0 times the SC
Testosterone (T)	**Basal hormone level in SC**	≥ 5 times the LOQ	≥ 16 times the LOQ
	Induction (10 µM Forskolin)	≥ 1.5 times the SC	≥ 1.5 times the SC
	Inhibition (1 µM prochloraz)	≤ 0.5 times the SC	≤ 0.2 times the SC

[a] QC = Quality Control,
[b] SC: Solvent Control
[c] LOQ: Limit of Quantification

2.1.2. LC-MS/MS method

Selectivity - The method was considered as selective since no peaks were observed before and after peaks of E2 and T. The chromatographic separation started with E2 in negative mode at 5.80 minute, followed by T in positive mode at 5.87 minute (Figure 2).

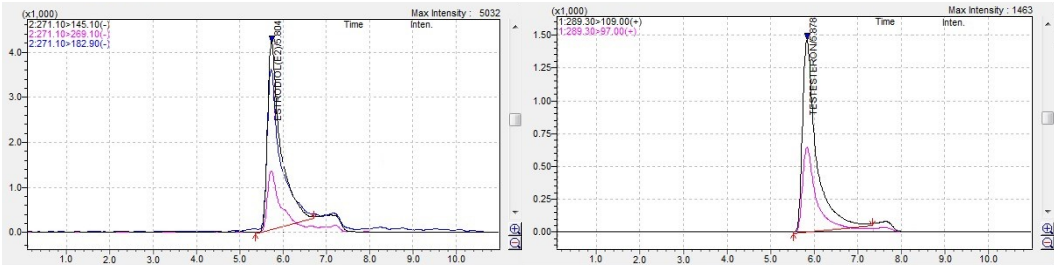

Figure 2. Representative chromatograms of spiked E2 and T (1 ng/ml) in the media.

Linearity - Calibration curves of the LC–MS/MS method were linear (r2 = 0.968 – 0.996) over a concentration range of 0.025–10 ng/ml for E2 and T.

Limit of Detection (LOD) and Limit of Quantitation (LOQ) - The detection limits (LODs; Signal (S) / Noise (N) = 3) were 0.004 ng/ml for E2 and 0.001 ng/ml for T. Additionally, the quantification limits (LOQs; S/N = 10) were 0.015 ng/ml for E2 and 0.005 ng/ml for T (Table 2).

Table 2. Determination coefficients (R2), LOD and LOQ of E2 and T.

	Correlation Coefficients (R^2)	LOD (ng/ml)	LOQ (ng/ml)
Estradiol (E2)	0.996	0.004	0.015
Testosterone (T)	0.968	0.001	0.005

Precision- T and E2 standards were spiked to the H295R media at both low (0.025 ng/ml) and high concentrations (0.1 ng/mL). The precision of the present LC–MS/MS method was acceptable. Between-day variations (CV%) were higher than the within-day variations for E2 (Table 3). Besides, within-day variation at 0.025 ng/ml concentration for T was higher than the between-day variation, however, CV% was unchanged in both cases for T at 0.1 ng/ml (Table 4).

Table 3. Quality control parameters for LC-MS/MS analysis of estradiol measurements assay in H295R cells. Two different concentrations of QCs were run in 6 replicates for each within-day and between-day repeatability experiments. Coefficient of variation was calculated as division of the standard deviation by mean. % Recovery calculated according to the formula given above. n: number of actual readings for each QC.

	Within-day Repeatability (n=6)		Between-day Repeatability (n=6)	
Estradiol (E2)	Low concentration (0.025ng/ml)	High concentration (0.10ng/ml)	Low concentration (0.025ng/ml)	High concentration (0.10ng/ml)
Average (ng/ml)	0.0252	0.106	0.0260	0.107
StD[a]	0.00098	0.00136	0.0015	0.0021
%CV[b]	3.91	1.28	5.96	2.04
% Recovery	74.44		88.80	

[a] StD = Standard Deviation,
[b] CV = Coefficient of Variation

Table 4. Quality control parameters for LC-MS/MS analysis of testosterone measurements assay in H295R cells. Two different concentrations of QCs were run in 6 replicates for each within-day and between-day repeatability experiments. Coefficient of variation was calculated as division of the standard deviation by mean. % Recovery calculated according to the formula given above. n: number of actual readings for each QC.

	Within-day Repeatability (n=6)		**Between-day Repeatability (n=6)**	
Testosterone (T)	Low concentration (0.025ng/ml)	High concentration (0.10ng/ml)	Low concentration (0.025ng/ml)	High concentratin (0.10ng/ml)
Average (ng/ml)	0.0024	0.105	0.0237	0.103
StD[a]	0.0011	0.0024	0.0012	0.0024
%CV[b]	6.94	2.30	5.12	2.33
% Recovery	77.27		80.46	

Recovery- Mean recovery data, which express the repeatability of the extraction method, are given in Table 3 and Table 4. Recoveries were between 74% and 89% for E2 and T.

2.2. Effects of references on E2 and T by LC-MS/MS

Forskolin, as a hormone inducer, increased E2 and T levels with a dose-dependent manner at 0.1-3 µM concentrations (Figure 3). As a known inhibitor of steroidogenesis, prochloraz decreased E2 and T levels dose dependently when compared to the 1 µM forskolin as control group, which was also added to wells for basal hormone induction in H295R cells (Figure 4).

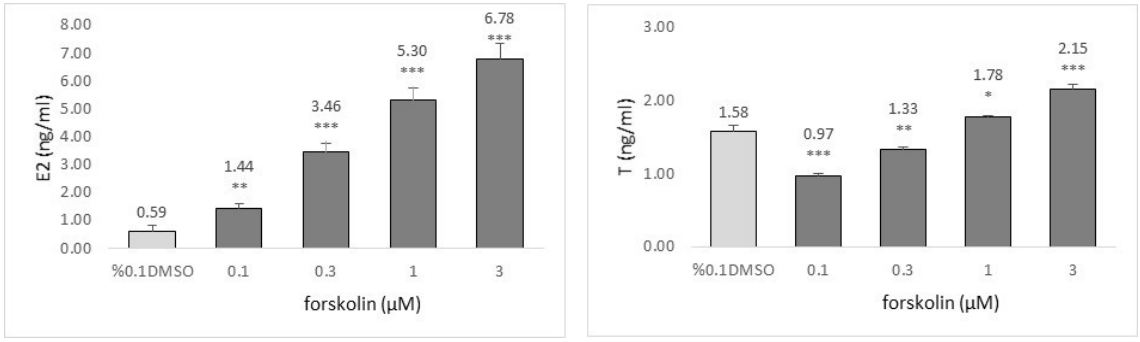

Figure 3. The effects of forskolin on E2 and T levels measured with LC-MS/MS. Bars represent means of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

Figure 4. The effects of prochloraz on E2 and T levels measured with LC-MS/MS. For basal hormone induction forskolin 1 µM was added to all wells. Bars represent means of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

In addition to forskolin and prochloraz as reference compounds, the effects of letrozole and ketoconazole on E2 and T were also tested, as a known aromatase inhibitor [25], and a general steroidogenesis inhibitor [21], respectively. E2 levels could not be detected due to dramatic aromatase enzyme inhibition in cells treated with letrozole. Compared to forskolin 1 µM, T levels were decreased by letrozole (Figure 5). Moreover, E2 and T levels was under the limit of detection in ketoconazole treated cells since it is known to inhibit the entire pathway dramatically (data not shown).

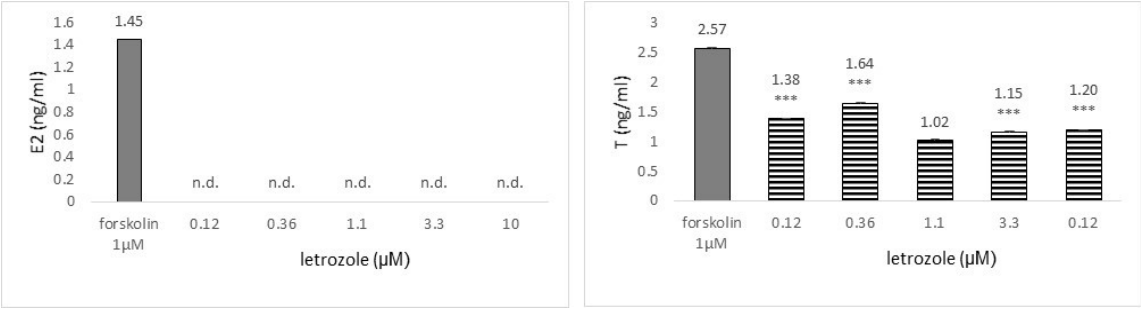

Figure 5. The effects of letrozole on E2 and T levels measured with LC-MS/MS. For basal hormone induction forskolin 1 µM was added to all wells. Bars represent means of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

2.3. Effects of melatonin and its analogues on E2 and T by LC-MS/MS

To induce basal hormone production, forskolin 1 µM was added to the incubation media in the presence of all tested compounds. Compared to the control group (1 µM forskolin alone), the level of E2 was decreased in cells exposed to MLT (3.3-90 µM) in a concentration-dependent manner (Figure 6A). MLT also caused a significant decrease in T production in H295R cells (Figure 7A). M6 reduced E2 level at all concentrations (Figure 6B). It showed stronger inhibitory effects at lower (0.01 µM) and higher (0.37, 1.1 and 3.3 µM) concentrations. Moreover, M6 reduced T levels at 0.01, 0.37, 1.1 and 3.3 µM (Figure 7B) but increased at 0.4 and 0.12 µM. These data show that M6 might have a biphasic effect on T production.

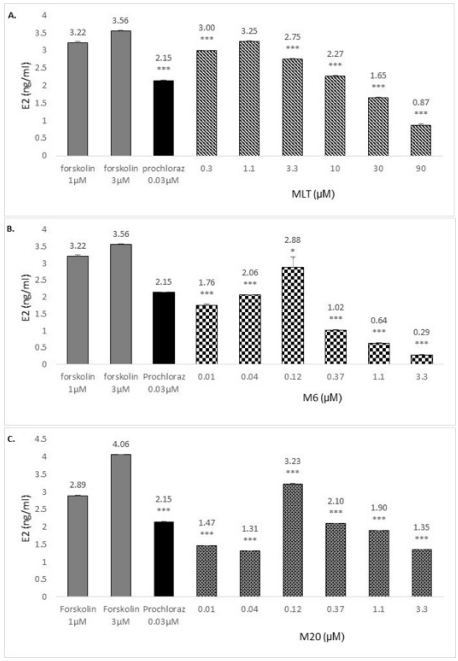

Figure 6. The effects of melatonin (A), M6 (B) and M20 (C) on E2 level measured with LC-MS/MS. Bars represent means of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

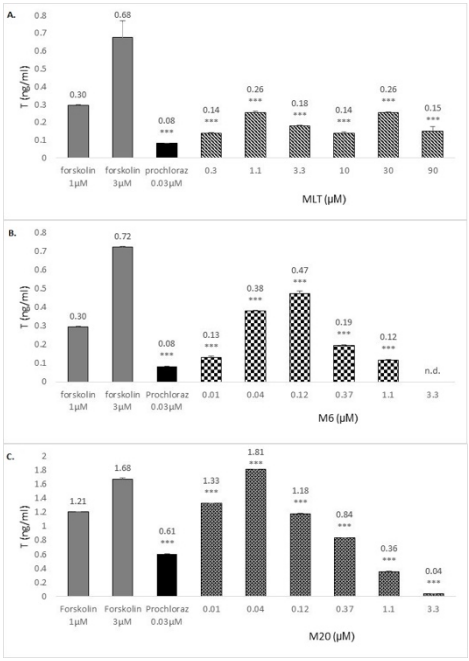

Figure 7. The effects of melatonin (A), M6 (B) and M20 (C) on T level measured with LC-MS/MS. Bars represent means of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

Compared to forskolin 1 µM, E2 level was decreased with M20 treatment at all tested concentrations except 0.12 µM (Figure 6C). The severity of this effect was also dose dependent as in M6. While it caused an increase in T level at 0.01 and 0.04 µM concentrations, hormone levels were decreased at higher concentrations of M20 (Figure 7C). These results show that this compound can also has biphasic effect on T hormone as in M6.

2.4. Effects of test compounds on cell viability

As stated in the OECD test guideline of Steroidogenesis Assay, the effects of the compounds on hormone levels should not be evaluated if the cell viability is below 80%. Therefore, effects of reference and test compounds on H295R cell viability was tested with MTT assay. None of the reference substances (forskolin, prochloraz, letrozole and ketoconazole) had cytotoxic effects (data not shown). Among the test compounds, MLT did not decrease cell viability below 80% (Figure 8A). Moreover, M6 and M20 increased cell viability at all tested concentrations except M20 at 3.3 µM (Figure 8B and 8C).

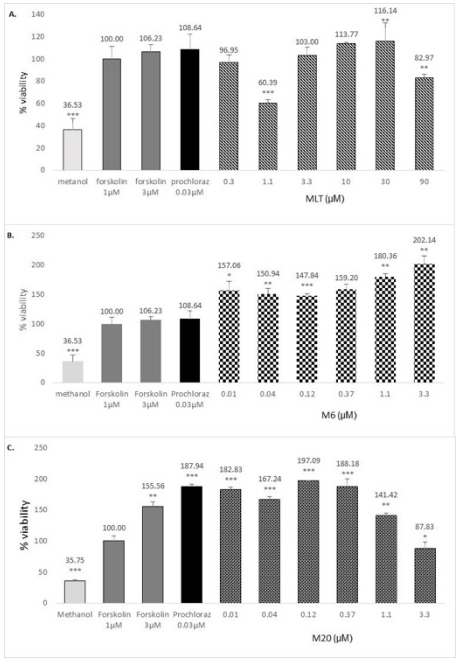

Figure 8. Effects of melatonin (A), M6 (B) and M20 (C) test compounds on cell viability. Bars represent means of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

3. DISCUSSION

OECD has been developed and validated H295R steroidogenesis assay to detect the endocrine disrupting/modulating effect of chemicals [17]. As H295R cells encode all key enzymes and contain all steroidogenesis pathway, the effects of chemicals on enzymes and hormone levels specially E2 and T which are the key steroid hormones of reproductive system, can be investigated by this in vitro cell system [23,26]. Consequently, the endocrine modulating effects of chemicals not only on aromatase (CYP19) which converts T to E2, but also on the other enzymes of the pathway can be determined. In addition, by performing a viability assay, it is possible to evaluate whether the decrease in hormone levels is due to the cytotoxic effects of the chemicals or not. Use of this high throughput and cost-effective in vitro assay during the drug discovery and development process can provide an insight into a possible reproductive toxicity of the candidate compounds without any animal use [27]. In case of changes in hormone levels (increase/decrease), this effect of the candidate molecules can be reported as a targeted therapeutical (anticancer drugs etc.) or an adverse effect.

MLT is an endogenous hormone and regulates the circadian rhythm. Generally, it is used as a dietary supplement in sleep disorders and jetlag [28]. Furthermore, it has also been found to be effective in diabetes, oxidative stress related neurodegenerative diseases and hormone-related breast cancer [2,29]. Anticancer effect of MLT in ER (+) breast cancer cells was reported to result from its inhibitory effect on aromatase and antagonising effect on ER [30-32]. MLT is reported to exert its antiproliferative effect through MLT receptor 1 (MT1) and MT2; by increasing the expression of these receptors and activating various signal transduction pathways [32]. Because of the short half-life and rapid metabolic inactivation of MLT, new MLT analogues have been synthesized and suggested for their beneficial health effects [12,13]. Previously, our group showed aromatase inhibitory effects of some novel MLT analogues on MCF7, ER (+) breast cancer cells. In the present research, possible effect of these MLT analogues on total steroidogenesis pathway is aimed to be detected by using human adrenocarcinoma H295R cells. T and E2 levels, as the end products of the steroidogenesis pathway, were measured by LC-MS/MS after performing partial validation of the method by using forskolin (as a pathway inducer), prochloraz (as a pathway inhibitor), letrozole (as an aromatase inhibitor) and ketoconazole (as a pathway inhibitor) as reference compounds [21,33]. The effects of these reference compounds on steroidogenesis (Figure 3, 4 and 5) were confirmed in the present study.

Blood MLT concentration is reported to be in the range of 10 – 120 pg/ml physiologically depending on the time of the day [34]. Therefore, MLT analogues were tested at physiological concentrations and higher concentrations to mimic the blood concentrations during dietary supplement usage. MLT decreased E2 and T levels and its inhibitory effect on E2 was dose dependent (Figure 6A and 7A). Despite the increased cell viability (Figure 8B), both M6 and M20 decreased E2 levels significantly with a dose dependent manner at all

tested concentrations except 0.12 µM (Figure 6B and 6C) and exerted biphasic effects on T levels (Figure 7B and 7C). These data indicate that both analogues inhibit aromatase enzyme significantly at low concentrations (0.01 and 0.04 µM; which are in the range of physiological concentrations) that cause a decrease only on E2 levels. It can be interpreted that besides aromatase inhibition, M6 and M20 also induce the previous enzymes of steroidogenesis pathway at 0.01 and 0.04 µM that cause an increase in T levels at the same time. Moreover, these MLT analogues decreased both E2 and T levels at higher tested concentrations (0.37, 1.1 and 3.3 µM) which indicate that both compounds inhibit the enzymes in early steps of the steroidogenesis pathway. These data demonstrate that as drug candidates the dose selection is essential for these MLT analogues and they may disrupt the steroidogenesis pathway with a non-monotonic dose response (NMDR) curve as it is for EDCs and natural hormones [35,36]. According to these results, MLT and its analogues (M6 and M20) should be used carefully in sleep disorders or oxidative stress related diseases because of their possible endocrine related effects, and dose should be adjusted especially for pregnant women as the fetus is known to be vulnerable to endocrine modulation. However, their potential endocrine modulating effects, namely inhibition of estrogen synthesis, can be used to design and develop potential anticancer drugs for hormone-dependent breast cancer.

Between numerous heterocyclic molecules, nitrogen-containing heterocycles have been widely examined as they establish the essential structures of many pharmacologically related molecules and have been observed to be effective against different types of cancers. Since indole is an active molecule, it has been a greatly advantaged structure for the target-based approach and progress of anticancer agents.

Some 3-substituted indole derivatives like M6 and M20 have been established to display significant anticancer activities. Suppression of cell proliferation of human colon carcinoma (HT-29), human ovarian adenocarcinoma (SK-OV-3), and c-Src kinase activity [37]. Similar indole derivatives were investigated for cytotoxic activity against various cancer cell lines. It is significant that indole derivatives substituted on the 3rd position with aromatic rings (particularly halogen containing) were found that they have significantly better activity [38]. These literature findings lead us to the assumption that the two analogous compounds used in our study are active due to the aromatic and halogen-containing aromatic rings they carry. Depending on our previous studies, especially chlorophenyl side chain containing indole derivative like M20 is more substantial [39-41]. Since this is a preliminary study on these molecules, it would be very appropriate to synthesize more melatonin analogue molecules for further investigation.

Although steroidogenesis assay is effective in evaluating effects of chemicals on all steps of the pathway this in vitro assay lacks hypothalamus-pituitary-gonad axis and chemicals effecting the pathway via this axis cannot be detected [17]. Another limitation of the H295R Steroidogenesis assay is the lack of metabolic capacity of the cells which make it impossible to determine the effects of possible active metabolites. Therefore, these drawbacks of the used assay should be kept in mind when interpreting the present data.

4. CONCLUSION

In conclusion, MLT and its two analogues, M6 and M20, are shown to interact with the enzymes in the early steps of the steroidogenesis pathway and they may have endocrine modulating potential through this mode of action. These MLT analogues seem to be promising candidates for prevention and treatment of ER (+) breast cancer because of their benefits of decreasing the estrogen levels. On the other hand, such endocrine related adverse effects of M6 and M20 should be considered when they are used as antioxidants in diseases related to oxidative stress, like neurodegenerative diseases. Further in vitro and in vivo studies should be performed to support the adverse endocrine modulating effects of these newly synthesized analogues.

5. MATERIALS AND METHODS

5.1. Chemicals

H95R cell line was purchased from American Type Culture Collection (ATCC). 17β-estradiol, Testosterone, Letrozole, Phosphate Buffered Saline and Thiazolyl Blue Tetrazolium bromide were purchased from Sigma Aldrich. DMEM/F12 containing L-glutamine without HEPES, Trypsin EDTA 0.25%, Penicillin-Streptomycin, were purchased from Gibco, Thermo Fisher. ITS premix and Nu Serum were purchased from Corning. Diethyl Ether and Methanol were obtained from Merck. Forskolin and Prochloraz were obtained from Cayman. Ketoconazole was obtained from Fisher Bioreagents, M6 and M20 compounds were synthesized in the previous study [42].

5.2. H295R cell culture

H295R cell lines were cultured in 75cm2 flasks with 10ml of Dulbecco's modified Eagle medium/Ham: F12 (DMEM/F12) without HEPES at 37°C with a 5% CO2 humidified atmosphere. Additionally, cell media

was supplemented with 2.5% Nu serum, 1% ITS (insulin, transferrin and selenium) + premix and 1% penicillin-streptomycin. Cells were passaged at approximately 80% confluency according to the ATCC H295R cell line passage protocol [24].

5.3. H295R steroidogenesis assay

Cells were seeded at 3×10^5 concentration in 1ml of complete media in 24 well plates and all assays were done between passages 5 - 10 as described in OECD test guideline 17. After 24 hours of incubation, reference (Forskolin: 0.1, 0.3, 1, 3 µM; Prochloraz: 0.03, 0.1, 0.3, 1 µM; Letrozole: 0.12, 0.36, 1.1, 3.3, 10 µM; Ketoconazole: 1, 5 µM) and test compounds (Melatonin: 0.3, 1.1, 3.3, 10, 30, 90 µM; M6: 0.01, 0.04, 0.12, 0.37, 1.1, 3.3 µM; M20: 0.01, 0.04, 0.12, 0.37, 1.1, 3.3 µM) in 0.1% DMSO were added and incubated for 48 hours. As positive control groups forskolin 3 µM (steroid hormone inducer) and prochloraz 0.03 µM (steroid hormone inhibitor) was used in all assays. For basal steroid hormone induction, forskolin 1 µM was added to each well. Each compound was tested in triplicates.

5.3.1. Measurement of hormone levels by LC-MS/MS method

LC-MS/MS conditions: LC-MS/MS analysis was carried out on a Shimadzu-8040 triple quadrupole mass spectrometer. Ionization was achieved by electrospray ionization (ESI) in positive and negative modes. The heat block and GC column temperature was maintained respectively at 400°C and 40°C. Nitrogen was used as collision gas. Chromatographic separation was achieved on a InertSustain C18 column (2.1 x 100 mm, 3 µm particle size). The column temperature was set at 40 °C. Mobile phase A consisted of water with 0.05% (v/v) ammonium hydroxide solution (25%), and mobile phase B consisted of methanol with 5% (v/v) ammonium hydroxide solution (25%). The flow rate of mobile phase was 0.3 ml per minute. The sample injection volume was 10 µl. T and E2 were detected and quantitated by multi-reaction monitoring (MRM). Quantifier and qualifier MRM-transitions are listed in Table 5.

Table 5. Multiple reaction monitoring (MRM) based analytical parameters for T and E2 steroid analytes measured in H295R cells, MRM transitions (Quantifier and Qualifier), analyte retention times, ionization for each analyte.

Analyte	Precursor Q1[a]/ Q3[b] (m/z)	Retention Time (min)	Ionization
Estradiol (E2)	271.1> 145.10/269.1 / 182.9	5.80	ESI-
Testosterone (T)	289.3> 97/109	5.87	ESI+

5.3.2. Preparation of calibration standards and quality control samples

Stock solutions of E2 and T (1 × 105 pg/ml) were prepared in H295R media. Eight calibration standard samples of E2 and T were prepared at concentrations of 25, 125, 250, 500, 1000, 2500, 5000 and 10000 pg/ml diluted with H295R media. Quality control (QC) samples were added at concentrations of 10 µM for forskolin and 1 µM for prochloraz to H295R cells. The calibration standards were prepared fresh and QC samples were stored at -20°C.

5.3.3. Sample preparation

To measure the E2 and T levels in H295R cells, 1ml of medium was collected from each well in eppendorf tubes. Hormones were extracted by liquid-liquid extraction method using 2.5 ml of diethyl ether. Samples were vortexed for 1 minute and then centrifuged at 1800 ×g. The organic phase was filtered with PTFE 0.22 µm membrane and transferred to new tubes and evaporated to dryness under a stream of nitrogen gas at 40°C. The samples were stored at -80°C. For hormone analysis residues were reconstituted in 150 µl mobile phase B and 10 µl of this sample was injected into the LC-MS/MS system.

5.4. MTT assay

After the collection of media for hormone analysis, the MTT assay was performed with cells in 24 well plates. Each well was washed with PBS, then complete medium and MTT solution were added in a ratio of 1:10. After 4 hours of incubation at 37°C with a 5% CO2 atmosphere, the formed formazan crystals in wells were dissolved with DMSO and the absorbance was measured in 550 nm using Varioskan Microplate Reader. Cell viability was calculated as described in OECD test guideline [17]. As a positive control group 70% methanol was used.

5.5. Statistical analysis

The data were analysed using the SPSS 25.0 program. Data were expressed as means ± SD. Statistical analysis was performed by using Student's t-test. Differences were considered significant $p<0.05$. p values are given in figure legends.

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REFERENCES

[1] Reiter RJ. The melatonin rhythm: both a clock and a calendar. Experientia. 1993; 49 (8): 654-664. https://doi.org/10.1007/BF01923947

[2] Kostoglou-Athanassiou I. Therapeutic applications of melatonin. Therapeutic advances in endocrinology and metabolism. 2013; 4(1): 13-24. https://doi.org/10.1177/2042018813476084

[3] Sánchez-Barceló EJ, Cos S, Mediavilla D, Martínez-Campa C, González A, Alonso-González C. Melatonin-estrogen interactions in breast cancer. Journal of pineal research. 2005; 38 (4): 217-222. https://doi.org/10.1111/j.1600-079X.2004.00207.x

[4] Cos S, González A, Martínez-Campa C, Mediavilla MD, Alonso-González C, Sánchez-Barceló EJ. Estrogen-signaling pathway: A link between breast cancer and melatonin oncostatic actions. Cancer detection and prevention. 2006; 30 (2): 118-128. https://doi.org/10.1016/j.cdp.2006.03.002

[5] Di Bella G, Mascia F, Gualano L, Di Bella L. Melatonin anticancer effects: review. International journal of molecular sciences. 2013; 14 (2): 2410-2430. https://doi.org/10.3390/ijms14022410

[6] Fraschini F, Demartini G, Esposti D, Scaglione F. Melatonin involvement in immunity and cancer. Biological signals. 1998; 7 (1): 61-72. https://doi.org/10.1159/000014529

[7] Hill SM, Frasch T, Xiang S, Yuan L, Duplessis T, Mao L. Molecular mechanisms of melatonin anticancer effects. Integrative cancer therapies. 2009; 8 (4): 337-346. https://doi.org/10.1177/1534735409353332

[8] Cos S, Martínez-Campa C, Mediavilla MD, Sánchez-Barceló EJ. Melatonin modulates aromatase activity in MCF-7 human breast cancer cells. Journal of pineal research. 2005; 38 (2): 136-142. https://doi.org/10.1111/j.1600-079X.2004.00186.x

[9] Chottanapund S, Van Duursen MBM, Navasumrit P, Hunsonti P, Timtavorn S, Ruchirawat M, Berg MVd. Anti-aromatase effect of resveratrol and melatonin on hormonal positive breast cancer cells co-cultured with breast adipose fibroblasts. Toxicology in vitro: an international journal published in association with BIBRA. 2014; 28 (7): 1215-1221. https://doi.org/10.1016/j.tiv.2014.05.015

[10] Martínez-Campa C, González A, Mediavilla MD, Alonso-Gonzalez C, Alvarez-Garcia V, Sanchez-Barcelo EJ, Cos S. Melatonin inhibits aromatase promoter expression by regulating cyclooxygenases expression and activity in breast cancer cells. British journal of cancer. 2009; 101 (9): 1613-1619. https://doi.org/10.1038/sj.bjc.6605336

[11] Grigg-Damberger MM, Ianakieva D. Poor quality control of over-the-counter melatonin: what they say is often not what you get. Journal of clinical sleep medicine: JCSM : official publication of the American Academy of Sleep Medicine. 2017; 13 (2): 163. https://doi.org/10.5664/jcsm.6434

[12] Rajaratnam SMW, Cohen DA, Rogers NL. Melatonin and melatonin analogues. Sleep Medicine Clincs. 2009; 4 (2): 179-193. https://doi.org/10.1016/j.jsmc.2009.02.007

[13] Ozcan-Sezer S, Ince E, Akdemir A, Ceylan ÖÖ, Suzen S, Gurer-Orhan H. Aromatase inhibition by 2-methyl indole hydrazone derivatives evaluated via molecular docking and in vitro activity studies. Xenobiotica; the fate of foreign compounds in biological systems. 2019; 49 (5): 549-556. https://doi.org/10.1080/00498254.2018.1482029

[14] Gurer-Orhan H, Suzen S. Melatonin, its metabolites and its synthetic analogs as multi-faceted compounds: antioxidant, prooxidant and ınhibitor of bioactivation reactions. Current medicinal chemistry. 2015; 22 (4): 490-499. http://doi.org/10.2174/0929867321666141215095259

[15] Shirinzadeh H, Neuhaus E, Ince-Erguc E, Tascioglu-Aliyev A, Gurer-Orhan H, Suzen S. New indole-7-aldehyde derivatives as melatonin analogues; synthesis and screening their antioxidant and anticancer potential. Bioorganic chemistry. 2020; 104219. https://doi.org/10.1016/j.bioorg.2020.104219

[16] Sanderson JT. The steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals. Toxicological sciences: an official journal of the Society of Toxicology. 2006; 94 (1): 3-21. https://doi.org/10.1093/toxsci/kfl051

[17] OECD Test No. 456: H295R Steroidogenesis Assay. https://read.oecd-ilibrary.org/environment/test-no-456-h295r-steroidogenesis-assay_9789264122642-en#page1. (accessed April 29, 2022).

[18] Albert O, Desdoits-Lethimonier C, Lesné L, Legrand A, Guille F, Bensalah K, Dejucq-Rainsford N, Jegou B. Paracetamol, aspirin and indomethacin display endocrine disrupting properties in the adult human testis in vitro. Human reproduction (Oxford, England). 2013; 28 (7): 1890-1898. https://doi.org/10.1093/humrep/det112

[19] Stukenborg JB, Mitchell RT, Söder O. Endocrine disruptors and the male reproductive system. Clinical endocrinology & metabolism. 2021; 35 (5), 101567. https://doi.org/10.1016/j.beem.2021.101567

[20] Craig ZR, Wang W, Flaws JA. Endocrine-disrupting chemicals in ovarian function: Effects on steroidogenesis, metabolism and nuclear receptor signaling. Reproduction (Cambridge, England). 2011; 142: 633-646. https://doi.org/10.1530/REP-11-0136

[21] Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sorensen B, Björklund E, Styrishave B. H295R cells as a model for steroidogenic disruption: A broader perspective using simultaneous chemical analysis of 7 key steroid hormones. Toxicology in vitro: an international journal published in association with BIBRA. 2012; 26 (2): 343-350. https://doi.org/10.1016/j.tiv.2011.12.008

[22] OECD Work Related to Endocrine Disrupters. https://www.oecd.org/chemicalsafety/testing/oecdworkrelatedtoendocrinedisrupters.htm (accessed April 29, 2022).

[23] Haggard DE, Karmaus AL, Martin MT, Judson RS, Setzer RW, Friedman KP. High-Throughput H295R Steroidogenesis Assay: Utility as an Alternative and a Statistical Approach to Characterize Effects on Steroidogenesis. Toxicological sciences: an official journal of the Society of Toxicology. 2018; 162 (2): 509-534. https://doi.org/10.1093/toxsci/kfx274

[24] ATCC. NCI-H295R. https://www.atcc.org/products/crl-2128 (accessed June 15, 2022).

[25] Higley EB, Newsted JL, Zhang X, Giesy JP, Hecker M. Assessment of chemical effects on aromatase activity using the H295R cell line. Environmental science and pollution research international. 2010; 17 (5): 1137-1148. https://doi.org/10.1007/s11356-009-0285-3

[26] Hecker M, Giesy JP. Novel trends in endocrine disruptor testing: The H295R Steroidogenesis Assay for identification of inducers and inhibitors of hormone production. Analytical and bioanalytical chemistry. 2008; 390 (1): 287-291. https://doi.org/10.1007/s00216-007-1657-5

[27] Maglich JM, Kuhn M, Chapin RE, Pletcher MT. More than just hormones: H295R cells as predictors of reproductive toxicity. Reproductive toxicology. 2014; 45: 77-86. https://doi.org/10.1016/j.reprotox.2013.12.009

[28] Chase JE, Gidal BE. Melatonin: Therapeutic use in sleep disorders. The Annals of pharmacotherapy. 1997; 31 (10): 1218-1226. https://doi.org/10.1177/106002809703101015

[29] Peschke E, Stumpf I, Bazwinsky I, Litvak L, Dralle H, Mühlbauer E. Melatonin and type 2 diabetes – a possible link?. Journal of pineal research. 2007; 42 (4): 350-358. https://doi.org/10.1111/j.1600-079X.2007.00426.x

[30] Kiefer T, Ram PT, Yuan L, Hill SM. Melatonin inhibits estrogen receptor transactivation and cAMP levels in breast cancer cells. Breast cancer research and treatment 2002; 71 (1): 37-45. https://doi.org/10.1023/A:1013301408464

[31] Cos S, Fernandez R, Güezmez A, Sãnchez-Barcelo EJ. Influence of melatonin on ınvasive and metastatic properties of mcf-7 human breast cancer cells. Cancer research. 1998; 58 (19): 4383-4390.

[32] Hill SM, Belancio VP, Dauchy RT, Xiang S, Brimer S, Mao L, Hauch A, Lundberg PW, Summers W, Yuan L, Frasch T, Blask DE. Melatonin: an inhibitor of breast cancer. Endocrine-related cancer. 2015; 22 (3): R183-R204. https://doi.org/10.1530/ERC-15-0030

[33] Hecker M, Hollert H, Cooper R, Vingguard AM, Akahori Y, Murphy M, Nellemann C, Higley E, Newsted J, Laskey J, Buckalew A, Grund S, Maletz S, Giesy J, Timm G. The OECD validation program of the H295R steroidogenesis assay: Phase 3. Final inter-laboratory validation study. Environmental science and pollution research international. 2018; 25 (15), 15265. https://doi.org/10.1007/s11356-010-0396-x

[34] Tordjman S, Chokron S, Delorme R, Charrier A, Bellissant E, Jaafari N, Fougerou C. Melatonin: pharmacology, functions and therapeutic benefits. Current neuropharmacology. 2017; 15 (3): 434. http://doi.org/10.2174/1570159X14666161228122115

[35] Darbre PD. Endocrine Disruption and Human Health. 1st ed, Reading, UK 2015.

[36] Lagarde F, Beausoleil C, Belcher SM, Belzunces LP, Emond C, Guerbet M, Rousselle C. Non-monotonic dose-response relationships and endocrine disruptors: A qualitative method of assessment. Environmental health: a global access science source. 2015; 14 (1): 1-15. http://doi.org/10.1186/1476-069X-14-13

[37] Kaur K, Jaitak V. Recent Development in Indole Derivatives as Anticancer Agents for Breast Cancer. Anticancer Agents Med Chem. 2019;19(8):962-983. http://doi.org/10.2174/1871520619666190312125602

[38] Sidhu JS, Singla R, Mayank, Jaitak V. Indole Derivatives as Anticancer Agents for Breast Cancer Therapy: A Review. Anticancer Agents Med Chem. 2015;16(2):160-73. http://doi.org/10.2174/1871520615666150520144217

[39] Ozcan-Sezer S, Ince E, Akdemir A, Ceylan ÖÖ, Suzen S, Gurer-Orhan H. Aromatase inhibition by 2-methyl indole hydrazone derivatives evaluated via molecular docking and in vitro activity studies. Xenobiotica. 2019 May;49(5):549-556. https://doi.org/10.1080/00498254.2018.1482029

[40] Shirinzadeh H, Neuhaus E, Ince-Erguc E, Tascioglu-Aliyev A, Gurer-Orhan H, Suzen S. New indole-7-aldehyde derivatives as melatonin analogues; synthesis and screening their antioxidant and anticancer potential. Bioorg Chem. 2020 Nov;104:104219. https://doi.org/10.1016/j.bioorg.2020.104219

[41] Shirinzadeh H, Ince E, Westwell AD, Gurer-Orhan H, Suzen S. Novel indole-based melatonin analogues substituted with triazole, thiadiazole and carbothioamides: studies on their antioxidant, chemopreventive and cytotoxic activities. J Enzyme Inhib Med Chem. 2016 Dec;31(6):1312-21. https://doi.org/10.3109/14756366.2015.1132209

[42] Öztürk-Ceylan Ö, MSc Thesis. Synthesis and biological activitie evaluation of new antimicrobial indole derivatives. Department of Biotechnology, Enstitute of Biotechnology, Ankara University, Keçiören, Ankara, Turkey, 2015.